

In vitro effects of selected medicinal plants shortlisted for clinical use in the Brazilian public health system in CYP3A4 mRNA gene expression, glutathione levels, and P-glycoprotein activity and their implications for herb-drug interactions.

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DECLARATION

I, André Luís Dias Araujo Mazzari confirm that the work presented in this thesis is my own. Where information has been delivered from other sources, I confirm that this has been indicated in the thesis.

Signed:this day.....2017

Abstract

The Brazilian Unified Public Health System (SUS) shortlisted various plant species of interest (RENISUS) for future clinical use. However, very little is known about their effects on metabolic and transporter proteins, which could potentially lead to herb-drug interactions (HDI).

To evaluate this, we conducted *in vitro* preclinical studies on twenty-four plant extracts to disclose their effects on CYP3A4 mRNA gene expression, intracellular glutathione (GSH) levels, inhibition of γ -glutamyl transferase (GGT) in HepG2 cells and P-glycoprotein (P-gp) activity in vincristine resistant Caco-2 (Caco-2 VCR) cells. We also investigated whether four Brazilian native species were able to activate the human pregnane X receptor (hPXR) in transiently co-transfected HeLa cells.

This preclinical research showed that all but two plant extracts were able to modulate at least one of the selected targets. CYP3A4 mRNA gene expression in HepG2 cells was significantly affected by half of the extracts. The antagonistic effect of *Solanum paniculatum* L. on hPXR could explain its ability to inhibit CYP3A4. GSH levels were affected by 80% of the extracts. There was depletion of intracellular GSH levels by *Cordia verbenacea* A. DC., *Costus spicatus* (Jacq.) Sw., *Persea americana* Mill., *Salix alba* L., *Schinus terebinthifolia* Raddi and *Syzygium jambolanum* (Lam.) DC. accompanied because of the inhibition of GGT activity. P-gp activity was modulated in a significant manner by 17% of the extracts.

The approaches used for the conduction of *in vitro* preclinical studies in herbal medicines revealed a series of challenges faced especially by academics in order to anticipate cases of HDI. Clinicians have also to consider the presence of intrinsic factors such as genetic polymorphisms in each patient. The possible presence of undesirable interactions between RENISUS herbal medicines and essential drugs in SUS need eventually be clinically confirmed to attest our observed *in vitro* effects.

Dedication

To dad, my big star. Thanks for inspiring me in each and every step I walk in my life.
Thanks to mom (Graça), sister (Vanessa) and Laika for supporting me on this journey.
I love you all.

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List of abbreviations

Acetyl-CoA	Acetyl-coenzyme A
ADME	Absorption, Distribution, Metabolism, and Elimination
ADR	Adverse drug reaction
AE	Adverse events
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANVISA	Agência Nacional de Vigilância Sanitária (National Health Surveillance Agency)
APS	Adenosine-5'-phosphosulphate
AST	Aspartate aminotransferase
BBB	Blood-brain barrier
BCS	Biopharmaceutics Classification System
BP	Brazilian Pharmacopoeia
BSO	Buthionine sulfoximine
Caco-2 VCR	Vincristine resistant Caco-2 cells
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
CGEN	Conselho de Gestao do Patrimonio Genetico (Genetic Heritage Council)
CNMM	Centro Nacional de Monitorização de Medicamentos (National Centre for Drug Monitoring)
CNS	Central nervous system
CYP	Cytochrome P450
DCIP	Desmethyldomipramine
DDI	Drug-drug interactions
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTB	5-5'-dithiobis(2-nitrobenzoic acid)
dsDNA	Double-stranded DNA
EA	Eventos adversos (adverse events)
FBS	Foetal bovine serum
FDA	Food and Drug Administration
Fiocruz	Fundação Oswaldo Cruz (Oswaldo Cruz Foundation)
GCL	Glutamate-cysteine ligase
GGT	Gamma-glutamyl transferase
GLU	Glucuronic acid
Gly-Gly	Glycyl-glycine
GpNA	γ -glutamyl- <i>p</i> -nitroanalide
GPx	Glutathione peroxidases
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione-S-transferase

HCl	Hydrochloric acid
HDI	Herb-drug interactions
HDL	High-density lipoprotein cholesterol
HeLa	Henrietta Lacks
HH	Human Hepatocytes
HLM	Human Liver Microsomes
HNF α	Hepatocyte nuclear factor α
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
hPXR	Human nuclear pregnane X receptor
IBAMA	Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Brazilian Institute of Environment and Renewable Natural Resources)
LA	Liquirtin apioside
LBD	Ligand-binding domain
LDC	Lead Drug Candidate
LDL	Low-density lipoprotein cholesterol
M-MLV	Moloney murine Leukaemia Virus
M-MLV RT	Moloney murine Leukaemia Virus Reverse Transcriptase
MDR1	Multi-drug resistance 1
MNTC	Maximum non-toxic concentration
mRNA	Messenger RNA
MTA	Material transfer agreement
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NAPQI	N-acetyl-p-benzoquinoneimine
NAT	N-acetyltransferase
NOTIVISA	Sistema de Notificações em Vigilância Sanitária (National notification System for Adverse Events and Technical Complaints)
NPR	Natural products reagent
NR	Neutral Red
NSAID	Nonsteroidal anti-inflammatory
P-gp	P-glycoprotein
PAPS	3'-phosphoadenosine-5'-phosphosulphate
PBS	Phosphate saline buffer
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PGC-1 α	Peroxisome proliferator-activated receptor γ coactivator-1- α
PK	Pharmacokinetic
PNPIC	Política Nacional de Práticas Integrativas e Complementares (National Policy of

	Integrative and Complementary Practices)
qPCR	Quantitative PCR
QT	Queixas técnicas (Technical complaints)
REFARGEN	Rede Nacional de Farmacogenética (Brazilian National Pharmacogenetics/Pharmacogenomics Network)
RENAME	Relação Nacional de Medicamentos Essenciais (Brazilian national essential medicines list)
RENISUS	Relação Nacional de Plantas Medicinais de Interesse ao SUS (Brazilian National List of Medicinal Plants of Interest to the Unified Health System)
Rf	Retention factor
Rh-123	Rhodamine 123
RIF	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT-PCR	Real-time polymerase chain reaction
RT-qPCR	Real-time quantitative PCR
RXR α	Retinoid X receptor α
SAM	S-adenosylmethionine
SHP	Small heterodimer partner
SJW	Saint John's Wort
SMRT	Silencing mediator of retinoid and thyroid hormone
SRB	Sulphorhodamine B
SRC-1	Steroid receptor coactivator-1
SSA	Sulfosalicylic acid
SULT	Sulphotransferases
SUS	Sistema Único de Saúde (the Brazilian Unified Health System)
T3	Triiodothyronine
TC	Technical complaints
TCA	Trichloroacetic acid
TCM	Traditional Chinese Medicine
UCL	University College London
UDPGA	Uridine 5'-diphosphate-glucuronic acid
UGT	UDP-glucuronosyltransferase
UnB	University of Brasília
UV-Vis	Ultraviolet/visible
VLDL	Very low-density lipoprotein cholesterol
WHO	World Health Organization
XREM/PXRE	Xenobiotic responsive enhancer molecule/PXRE

β -NADPH

β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate

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- Contribution of Genetic Polymorphism to Potential Herb-Drug Interactions Among the Brazilian Population. International Conference on Toxicogenomics and Drug Monitoring. August 25 – 27, 2015. Valencia, Spain. **Abstract published in “Journal of Drug Metabolism & Toxicology”**. DOI: 10.4172/2157-7609.S1.002
- Modulation of P-glycoprotein Efflux Pump Activity in Vincristine Resistant Caco-2 cells by Brazilian Traditional Medicine. Asian Congress on Biotechnology. November 15 – 19, 2015. Kuala Lumpur, Malaysia.
- Preclinical Pharmacokinetic Profile of Four Traditional Medicinal Plants. 11th International ISSX Meeting. June 12 – 16, 2016. Busan, South Korea. **Abstract to be published in “Drug Metabolism Reviews”**
- Preclinical Pharmacokinetic Profile of Four native Brazilian Medicinal Plants. 9th Joint Natural Products Conference 2016. July 24-27, 2016. Copenhagen, Denmark. **Abstract to be published in “Planta Medica”**

1 GENERAL INTRODUCTION

1.1 HERBAL MEDICINES: TRADITIONAL USE AND SAFETY CONCERNS

According to the World Health Organization (WHO), it is estimated that up to four billion people depend on medicinal plants for their primary health care due to poverty or lack of access to modern medicine, this constitutes between 65 and 80% of the world's population in developing countries (Silveira et al., 2008). In contrast, in the developed world, herbal medicines are mostly used due to the belief that they promote healthy living (Ekor, 2013).

The national policy on traditional medicines and regulation of herbal medicines report published in 2005 by WHO shows that up to 90% of the African population depend on traditional medicines, followed by India (70%) and China (40%) (WHO, 2005). Also, herbal medicines are used in primary health care in about 350 locations only in Brazil (Antonio et al., 2013). Neglected tropical diseases, such as Chagas disease and leishmaniasis, are endemic in many tropical countries. As such diseases are more likely to affect low-income populations private pharmaceutical companies do not invest in the development of new medications for such conditions. Therefore, in many developing countries, traditional knowledge on medicinal plants is a valuable resource to keep the population healthy (Ndjonka et al., 2013).

Although most of the herbal medicines traditionally used are known for their effectiveness on numerous conditions, safety concerns are rarely effectively disclosed to the population that uses them. Cases of acute toxicity caused by intake of herbal medicines, for example have led to acute hepatitis (Haller et al., 2002) and nephrotoxicity in patients (Asif, 2012). Also, chronic toxicity was found in herbal preparations such as the Nigerian DAS-77 (Afolabi et al., 2012). Cases of toxicity involving herbal medicines rarely impact the natural products industry. Despite the risks of consuming many herbal medicines (particularly with excessive chronic use), the general public continues to perceive such products as “natural” without being properly informed about the associated risks (Ekor, 2013).

The growth of manufactured drugs was accompanied by a series of events that questioned their actual safety; one of the most noted ones being the thalidomide case. When the pharmacological effects of thalidomide were first reported in 1956, regulations on the safety of new drugs were not as strict as they currently are. Cases of teratogenicity raised quickly due to the consumption of the drug by pregnant women, which led to the quick withdrawal of thalidomide from the market and also forced the UK and USA regulatory bodies to make changes in their drug safety regulations.

A remarkable case of the natural compound aristolochic acid led to a similar outcome as thalidomide. In the early 1990s the cases of nephropathy linked to Chinese herbs among Belgian women, this was due to the introduction of the Chinese species *Aristolochia* in a popular slimming regimen. Studies on aristolochic acid detected its potential nephrotoxic and carcinogenic effects in the body. Following these findings herbs containing aristolochic acid were banned for medicinal use in many other countries including Canada, Australia, Germany, and the UK (Cosyns, 2003). The thalidomide and aristolochic acid cases demonstrated the potential risks that a drug could have on a population. However, this is not the only concern of the pharmaceutical companies. The risks of adverse drug reactions (ADRs) due to drug interactions are also very relevant in terms of drug safety (Botting, 2002).

When drugs are taken in combination with other drugs, the chances of drug-drug interactions (DDI) are increased (Boobis et al., 2009). In addition to DDI several types of drug interactions have been identified and documented in the literature: drug-food, drug-chemical substance, drug-laboratory, and non-laboratory test (Mazzari et al., 2016). DDI occur at both pharmacodynamic (PD) and pharmacokinetic (PK) levels. PD interactions can be defined as how drugs influence each other's effects directly by a synergistic or antagonistic effect. For example, nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids lead to additive interactions and the possible PD interaction effect is an increased risk of gastric bleeding. PK interactions occur when a drug is able to alter the absorption, distribution, metabolism, and elimination (ADME) of another drug. An example of a PK interaction at the metabolic level is the

antidepressant fluvoxamine, which is a potent cytochrome P450 (CYP) 1A2 inhibitor. The drug can interact with theophylline, which is a substrate of this cytochrome isoform. As a consequence of their coadministration, the bioavailability of theophylline will be increased as well as its toxic effects (Cascorbi, 2012).

Drugs, as well as environmental pollutants, food additives and insecticides are exogenous substances to the body that are cleared by a series of chemical reactions to avoid their accumulation in the organism. Such substances are also called xenobiotics. Xenobiotics are defined as chemical entities to which an organism is exposed that are extrinsic to the normal metabolism of that organism. If xenobiotics are not metabolised, they can reach toxic concentrations in the body and thereby cause damage to tissues and organs (Croom, 2012).

Herbal medicines, like any other xenobiotic, are able to cause both PD and PK-ADME interactions if coadministered with conventional drugs, causing the so-called herb-drug interactions (HDI). PD interactions between *Piper methysticum* (Piperaceae) and the benzodiazepine alprazolam is just one of the numerous documented. Following the hospital admission of a 54-year-old man in a lethargic and disoriented state after being coadministered *Piper methysticum* and alprazolam, the case report published in 1996 suggested that *Piper methysticum* might have additive effects on benzodiazepines (Almeida and Grimsley, 1996). One of the most popular herbal medicines, St. John's wort (SJW) – *Hypericum perforatum* L. (Hypericaceae) - has been extensively studied regarding its absorption and metabolic HDI with several drugs, including contraceptives. In this case, the intake of SJW was found to increase the expression of intestinal P-glycoprotein (P-gp) and CYP3A4 in the liver, therefore potentially interfering with the action of drugs that are also metabolised by CYP3A4 (Murphy et al., 2005).

Many of the documented metabolic HDI cases that were caused by traditional, the ones involving SJW were highly relevant in helping scientists to identify the experimental targets that identified most of the HDI events that are currently known.

1.2 EVOLUTION OF HERB-DRUG INTERACTIONS RESEARCH AND THE INTRODUCTION OF METABOLIC AND TRANSPORTER EXPERIMENTAL TARGETS

Cases of HDI are well documented in the literature. We conducted a search on PubMed database using the keywords “herb-drug interactions”. This helps to understand the beginning and the development of this type of research over the years.

The first record of a publication on HDI was in 1967 (Figure 1-1). This single work assessed potential interactions between herbal medicines and anaesthetic drugs (Pellerano, 1967). Subsequent years were marked by a few publications in diverse subjects where HDI were mentioned but were the main scope of the research. In 1974, a publication on the interaction between cannabinoids and phenytoin (Chesher and Jackson, 1974) triggered more interest in the subject. In 1975, two of the four HDI publications were about cannabinoids (Hine et al., 1975, Hollister and Gillespie, 1975) and in 1978 the single publication found was also about interactions with cannabis (Singh and Das, 1978).

In 1990, the first work on HDI involving numerous herbal medicines was published by a Chinese researcher who evaluated the potential interactions between Western antihypertensive drugs and Chinese herbal drugs (Wang, 1990). From 1990 to 1993 more articles involving drug interactions with various herbal medicines were published as review articles (D'Arcy, 1991, D'Arcy, 1992, D'Arcy, 1993).

Interactions involving Chinese herbal medicines were again the subject of the only article published in 1995 according to our PubMed search (Tam et al., 1995). Two years later, in 1997, grapefruit (*Citrus paradisi* Macfad. - *Rutaceae*) and *Ginkgo biloba* L. (*Ginkgoaceae*) were investigated for their potential interactions with synthetic drugs (Ameer and Weintraub, 1997, Chermat et al., 1997) and in 1998 two more research articles (one again about drug interactions with grapefruit) were published (Miller, 1998, Fuhr, 1998).

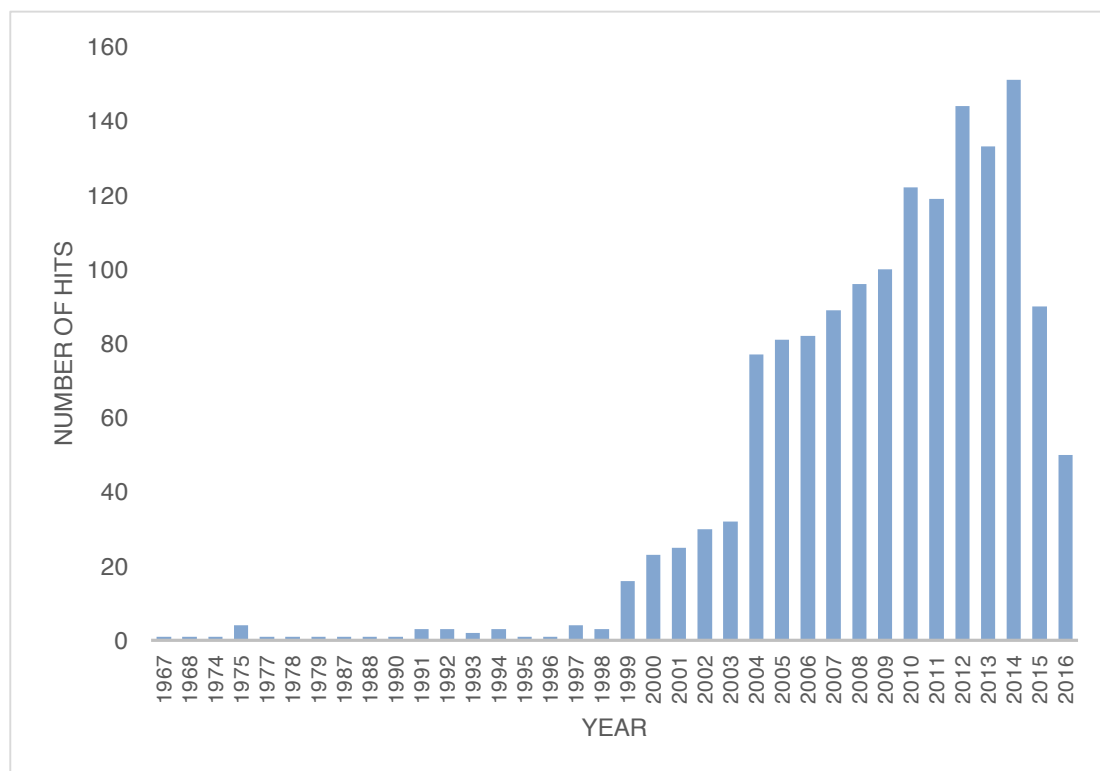


Figure 1- 1 Number of publications per year using the keyword “herb-drug interactions” on PubMed. Data was collected on 15 August 2016.

The highest number of publications on HDI from 1967 to 1998 was four, with an average of 1.06 articles per year. There was a sharp increase in the number of published articles from 1998 (three articles) to 1999 (sixteen articles), this is over a 500% increase in one year. The number of publications published in subsequent years grew exponentially reaching a peak in 2014 when 151 articles were published. Interestingly, a sharp drop can be observed in 2015 and during this search (August 2016), the number of published articles on HDI was almost three times less as compared to 2014.

In addition to the number of articles, it is also possible to observe an evolution of the type of HDI reports. Prior to 1999 most articles focused on single herbs, such as SJW. In the year 2000, about 35% of the total of publications on HDI were based exclusively on SJW interactions (Ingram et al., 2000, Mehta, 2000, Baede-van Dijk et al., 2000, McIntyre, 2000, Roots, 2000, Jordan, 2013). During the 00’s decade, SJW was the main subject of many HDI studies, mostly due to the publication of case reports involving the herbal medicine. For example, a case report published in 1999 showed that consumption of SJW together with other prescription antidepressants was able

to cause severe ADRs due to HDI, particularly in elderly people (Lantz et al., 1999). However, it was not clear how SJW could be causing such effects.

The first attempt to elucidate one of the possible interaction mechanisms of SJW was published in 2000, suggesting that the herbal medicine was able to induce CYP3A4 and consequently reduce the efficacy of certain drugs such as oral contraceptives (Roby et al., 2000). This was the starting point of the increased number of research articles evaluating how SJW could potentially cause interactions with various synthetic drugs, as most of them were known to be metabolised mainly by CYP3A4.

In addition to CYP3A4, other phase I metabolising enzymes such as the CYP isoforms CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 were becoming the subject of metabolic studies involving herbal medicines. The effects of SJW on P-gp and phase II metabolic enzymes, such as glutathione-S-transferase (GST) started to be also investigated. Herbal medicines have increasingly become the subject of metabolic and transporter studies using similar molecular targets as the ones used for synthetic drugs but with only one difference: such studies are not a regulatory requirement to market traditional herbal medicines in many countries (Tsay et al., 2016).

1.3 DRUG METABOLISM AND TRANSPORT: GENERAL

CONSIDERATIONS

Biotransformation is a process whereby a drug undergoes a series of processes for its conversion into metabolites with lower affinity characteristics. This system involves numerous mechanisms and enzymes that are ubiquitously present in the body (Ionescu and Caira, 2005). Some less lipid-soluble drugs can be cleared from the body unchanged, i.e. without being directly affected by phase I and phase II metabolism. However, other drugs remain in the body and their effects can still be felt for a long time (Baxter and Stockley, 2008).

Drug metabolism is normally divided into two phases: phase I metabolism (functionalisation reactions), which results in metabolites with higher polarity (usually inactive) and phase II metabolism (conjugative

reactions), which results in the phase I metabolites becoming even more polar (Figure 1-2). Phase I reactions prepare the drug for phase II metabolism by adding polar functional groups to the xenobiotic (Ionescu and Caira, 2005).

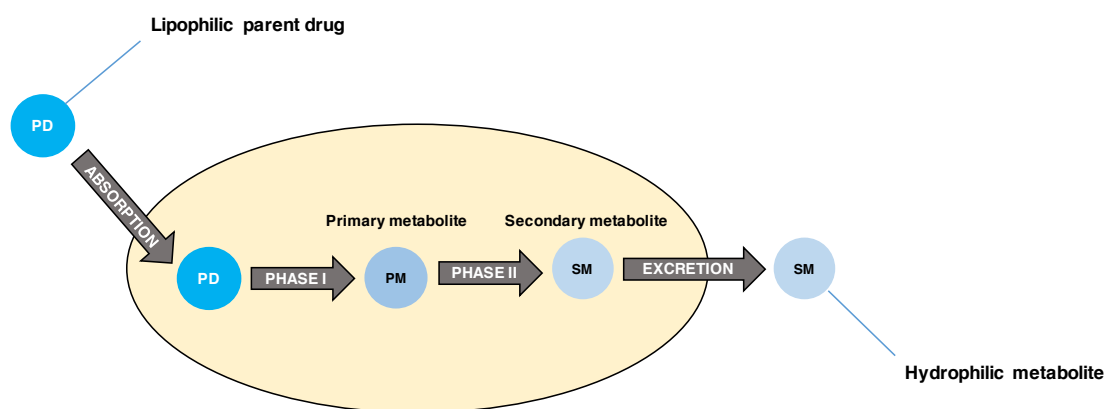


Figure 1- 2 Scheme of sequential drug metabolism

1.3.1 Phase I metabolism: cytochrome P450

Human drug-metabolising enzymes are present ubiquitously in the body. CYP450 is a family of enzymes responsible for the metabolism of various drugs. Over 50 human CYPs have already been isolated; the major ones found in the liver include CYP1A2, CYP2C9/19, CYP2D6 CYP2E1, and CYP3A4/5/7 (Figure 1-3) (Gibson and Skett, 2001). The whole CYP family is responsible for metabolising about 90% of commonly used drugs. The CYP 1, 2, and 3 families are the most abundant families of CYP metabolising enzymes and the CYP1A2, CYP2C, and CYP3A4 isoforms account for the metabolism of the majority of prescription drugs (Atkinson, 2012). Among the main isoforms, most of the currently marketed medicines share drug metabolism with CYP3A4, resulting in severe drug interactions (Ogu and Maxa, 2000).

The CYP monooxygenase enzymes are located in the smooth endoplasmic reticulum of the liver and other extrahepatic tissues. While most of the CYPs are located in the liver, extrahepatic metabolism also takes place in the kidneys, skin, gastrointestinal tract, and lungs for example. Numerous drugs are metabolised by CYP enzymes through oxidation reactions such as

aromatic and aliphatic hydroxylation, *N*-dealkylation, *O*-dealkylation, deamination, oxidation and sulfoxidation (Figure 1-4). Reduction, hydrolysis, and hydration are other examples of phase I reactions catalysed by the CYP (Ionescu and Caira, 2005). The product of these functional chemical reactions are compounds with chemically reactive functional groups that will be further targeted by phase II enzymes.

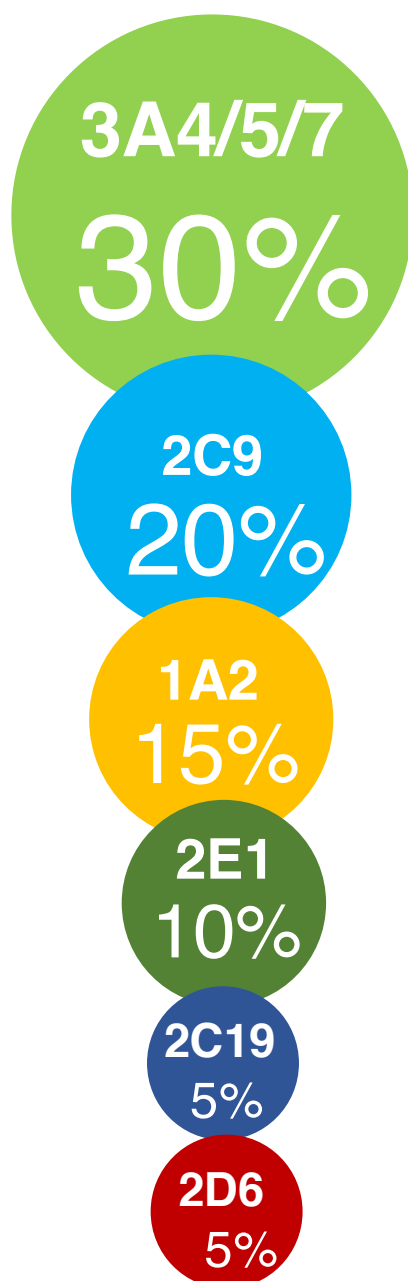
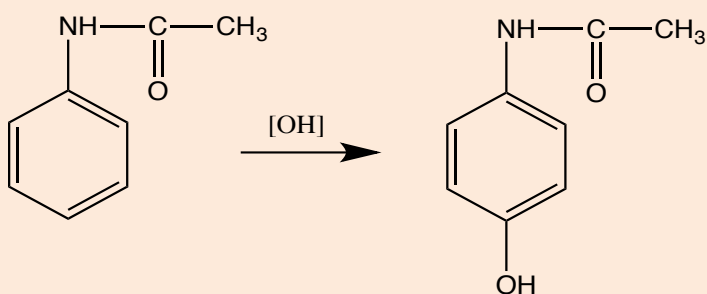


Figure 1- 3 Human liver CYPs represented by circles whose size and percentage approximate to their level of expression in human liver.

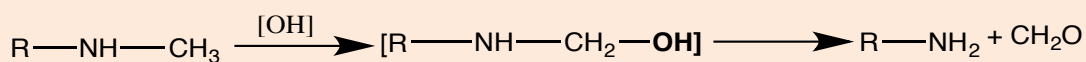
Aromatic hydroxylation



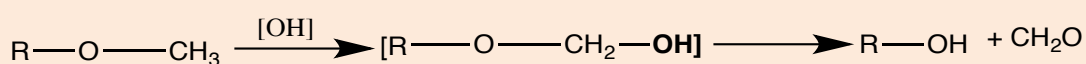
Aliphatic hydroxylation



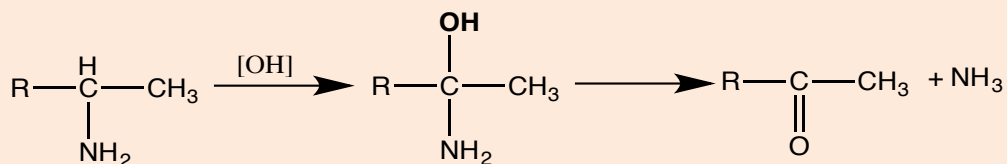
N-Dealkylation



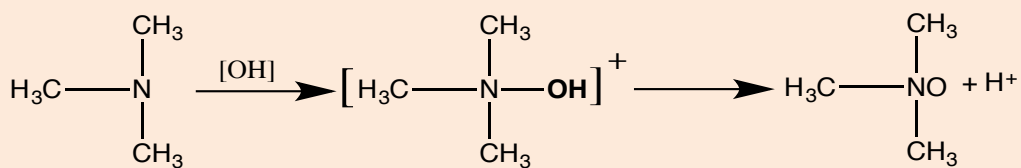
O-Dealkylation



Deamination



Oxidation



Sulphoxidation

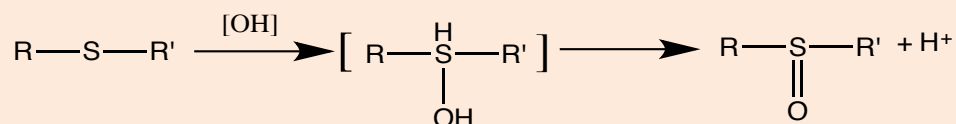


Figure 1- 4 Common reactions catalysed by CYP enzymes.

1.3.1.1 Main CYP isoforms

1.3.1.1.1 CYP1A2

This CYP isoform is mainly found in the liver (15%) and it metabolises almost 20% of the current therapeutic arsenal (Wang and Zhou, 2009). Examples of synthetic drugs, which are substrates for the CYP1A2, include the highly popular painkiller acetaminophen, the beta-blocker propranolol, the antidepressant clomipramine and the anticoagulant warfarin.

1.3.1.1.2 CYP2C9

CYP2C9 is the major CYP2C isoform found in the human liver (Mo et al., 2009) and it metabolises approximately 15% of clinical drugs, including the nonsteroidal anti-inflammatory (NSAID) ibuprofen, the antihypertensive losartan, the antidepressant fluoxetine, the antiepileptic phenytoin, and the anti-hypercholesterolaemic fluvastatin (Baxter and Stockley, 2008, Berka et al., 2011). Certain endogenous bioactive substances such as steroids, melatonin, retinoids, and arachidonic acid are also metabolised by this CYP isoform (Mo et al., 2009).

1.3.1.1.3 CYP2C19

CYP2C19 is not only involved in the metabolism of a range of drugs but it also plays a crucial role in the detoxification and inactivation of some potential carcinogens (Wang et al., 2013). CYP2C19 is responsible for the metabolism of about 10% of prescribed drugs, including the proton pump inhibitor omeprazole, the tricyclic antidepressant amitriptyline, the selective serotonin reuptake inhibitor fluoxetine, the benzodiazepine diazepam, and the barbiturate phenobarbital (Zhou et al., 2009).

1.3.1.1.4 CYP2D6

Although the level of expression of CYP2D6 in the human liver is only about 5%, this CYP isoform metabolises about 25% of all medications in the human liver (Ionescu and Caira, 2005). The beta-blockers propafenone and timolol, the antidepressant amitriptyline, the antipsychotics haloperidol and

risperidone, and the antihistamine chlorphenamine are examples of drugs that are metabolised by this CYP isoform (Zhou et al., 2009).

1.3.1.1.5 CYP2E1

CYP2E1 represents 10% of the total CYPs expressed in the human liver and it is well known for its involvement in the metabolism of ethanol to acetaldehyde, and accordingly it is rapidly induced after ethanol ingestion (Anzenbacher and Anzenbacherova, 2001). This CYP isoform is responsible for the activation of some carcinogens, procarcinogens, and toxicants and it metabolises mainly low-molecular-weight compounds. CYP2E1 also has the ability to produce reactive intermediates, leading to the formation of free radicals such as superoxide, hydroxyl radical, and lipid peroxides (Neafsey et al., 2009). Acetaminophen and the anaesthetic halothane are examples of CYP2E1 substrates (Tateishi et al., 1998, Wolf et al., 2007).

1.3.1.1.6 CYP3A

The most abundant subfamily of CYPs is CYP3A. It represents about 30% of the entire CYP450 enzymes in the liver and it is responsible for the biotransformation of more than 50% of therapeutic drugs. CYP3A's main isoforms found in the body are 3A4, 3A5, and 3A7. CYP3A5 is more often detected in adolescents than in adults, where it is hardly inducible. CYP3A4 is mostly glucocorticoid-inducible and CYP3A7 (found only in fetal livers) has a role in the hydroxylation of allylic and benzylic carbon atoms (Ionescu and Caira, 2005). Macrolide antibiotics, antiarrhythmics, benzodiazepines, immune modulators, HIV antivirals, antihistamines, calcium channel blockers, and HMG-CoA reductase inhibitors are examples of classes of medications metabolised by the CYP3A subfamily (Zhou et al., 2009). The probability of HDI with this isoform is high and therefore a particular attention to all CYP3A substrates should be given in order to avoid such interactions.

1.3.2 Phase II metabolism (conjugation reactions)

Phase II metabolism reactions (or conjugation reactions) occur when metabolic enzymes react with functional groups of a drug that were formed

during the phase I process. Endogenous species, such as a sugar or an amino acid, are added to the drug in order to increase the polarity to allow its elimination. The two main phase II biotransformation reactions are glutathione (GSH) conjugation and glucuronidation. However, the other conjugative reactions such as sulphation, methylation, and acetylation are also relevant (Atkinson, 2012). The main metabolising enzymes involved in phase II metabolism are listed in table 1-1.

Table 1- 1 The main phase II metabolic mechanisms and respective enzymes.

Phase II mechanism	Enzyme involved
Glucuronidation	UDP-glucuronosyltransferase (UGT)
Glutathione (GSH) conjugation	Glutathione-S-transferase (GST)
Sulphation	Sulphotransferases (SULT)
Methylation	Methyltransferases
Acetylation	CoA-S-acetyltransferase

1.3.3 Types of phase II reactions

1.3.3.1 *Glucuronidation*

Glucuronidation is a mechanism in which a glucuronide is formed by the reaction between the electrophilic C-1 atom of the pyranose acid ring of the co-factor UDPGA (uridine 5'-diphosphate-glucuronic acid) and the substrate catalysed by UGTs. UGTs are the most important phase II enzymes, and out of all the conjugation enzymes in the liver, they constitute the highest amount (35%) (Ionescu and Caira, 2005, Caira and Ionescu, 2006). The enzyme can be found in the kidneys, small intestine, lung, skin, adrenals, and spleen. This is the most important form of conjugation of xenobiotics with chemical groups such as alcohols, phenols, hydroxylamines, carboxylic acids (Figure 1-5), amines, sulphonamides, and thiols (Gibson and Skett, 2001).

UGTs exists in multiple isoforms (1A1, 1A3, 1A4, 1A6, 2B4, 2B7, 2B10, 2B11, and 2B15) and they are able to metabolise a wide range of compounds, hence providing the liver with the extensive capacity to glucuronidate numerous endogenous and xenobiotic compounds. An example of this process is:

UGT2B7, which catalyses the glucuronidation of opioids, and UGT1A6 and UGT1A1, which target primary amines and bilirubin respectively (Gibson and Skett, 2001).

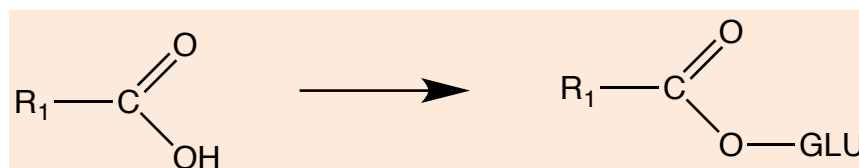


Figure 1- 5 Conjugation of a glucuronic acid (GLU) to a carboxylic acid group.

1.3.3.2 GSH conjugation

GSH is a tripeptide present in high concentrations in the liver. It has a protective role, removing toxic electrophilic compounds from the body (Ionescu and Caira, 2005). Conjugation with GSH is catalysed by GSTs, which are a family of enzymes (Alpha, Mu, Pi, Sigma, Theta, Zeta, Omega, and Kappa.) distributed in the body and especially in the liver cytosol (Figure 1-6). GSTs avoid the reaction of electrophilic compounds to nucleophilic ones in macromolecules such as proteins and nucleic acids. When the conjugate is formed, it has to undergo further metabolic reactions until the final product is eliminated from the organism in the form of mercapturic acid (Sies and Ketterer, 1988).

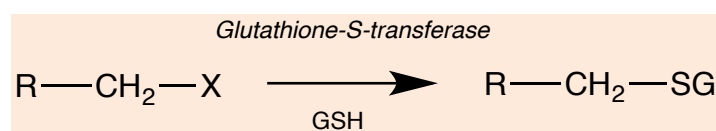


Figure 1- 6 Conjugation of glutathione (GSH) catalysed by the enzyme glutathione-S-transferase.

1.3.3.3 Sulphation, methylation, and acetylation

In addition to the two main liver conjugation reactions (GSH conjugation and glucuronidation), the xenobiotic transformation can also happen by sulphation (or sulphate conjugation), methylation (or methyl conjugation) and acetylation (Ionescu and Caira, 2005).

Sulphation is another phase II detoxification mechanism that is recognised as the major conjugation pathway for phenols, alcohols, amines and

thiols (Ionescu and Caira, 2005). Before the phase I metabolites undergo sulphate conjugation, the inorganic sulphate has to be activated via ATP to form adenosine-5'-phosphosulphate (APS) and consequently 3'-phosphoadenosine-5'-phosphosulphate (PAPS). SULTs will then catalyse the detoxification of essential drugs, such as salbutamol and acetaminophen by transferring a sulphuryl group from PAPS to an acceptor molecule (Gibson and Skett, 2001).

Methylation is the mainly metabolic pathway for endogenous compounds, but it can also be the route for many drugs and xenobiotics in general (Weinshilboum, 1988). Methyl conjugate reactions are only possible in the presence of the cofactor *S*-adenosylmethionine (SAM) and will result in the formation of *O*-methylated, *N*-methylated, and *S*-methylated products (Gibson and Skett, 2001).

The liver is the primary site for acetylation reactions, but the reaction can also happen in some extra hepatic sites, such as the spleen, lungs, and gut. To summarise, the *N*-acetyltransferases will catalyse the transferring of the co-factor acetyl-coenzyme A (acetyl-CoA) to aromatic amines and sulphonamides and form the polar metabolites (Ionescu and Caira, 2005).

1.3.4 The drug transporter P-glycoprotein

Drug-transporter proteins are known to allow xenobiotics to cross biological membranes, the most well-known one being P-gp. P-gp was first discovered in 1986 as a product of the multi-drug resistance gene (MDR1) in cancer cells, therefore reducing the intracellular accumulation of drugs (Watanabe et al., 2012).

This protein plays a role as an efflux pump, it pushes metabolites and drugs out of the cells which can result in absorption alterations (Williamson et al., 2009). P-gp contains two ATP-binding sites, where ATP will bind in presence of a P-gp substrate. As a consequence, ATPase is activated in order to hydrolyse ATP. The energy produced by the ATP hydrolysis will allow the P-gp to transport numerous substrates across cellular membranes (Watanabe et al., 2012). In the intestine, P-gp can be found on the apical surface of epithelial

cells. When a drug is taken up by an enterocyte, the substance can be either metabolised by CYP3A4 or pumped back into the lumen (Butterweck and Derendorf, 2008). Hence, oral delivery can be compromised (Butterweck et al., 2004).

1.4 INDUCTION AND INHIBITION OF DRUG METABOLISING ENZYMES

1.4.1 Induction of drug metabolising enzymes

As previously stated in section 1.2, studies on SJW demonstrate how the herbal medicine can act as a potent CYP3A4 inducer. As a consequence, drugs like oral contraceptives, which are metabolised by the same enzyme, will have their pharmacological effects altered. The increased metabolism of the drug due to high CYP3A4 activity will lead to a decreased blood concentration of the oral contraceptive and low pharmacological activity (Murphy et al., 2005). Consumption of metabolic enzyme inducers like SJW will, therefore, increase the chances of inefficacy of the coadministered drug in usual therapeutic doses due to HDI (Tarirai et al., 2010).

Another example of metabolic HDI involving CYP was published as a case report in 2015. The authors showed that the intake of noni juice, which is an herbal remedy made from the fruit of *Morinda citrifolia* L. (*Rubiaceae*), is able to induce CYP2C9 and interact with the anti-epileptic drug phenytoin by decreasing its bioavailability in a 49-year-old man (Kang et al., 2015).

The main types of drug induction are substrate-dependant induction, receptor-mediated and inhibitor-mediated interaction. Substrate-dependant induction is where the herbal drug influences the metabolism and duration of action of numerous other drugs. Receptor-mediated is characterized by interactions with important regulator pathways, which is the case of the human nuclear pregnane X (hPXR) and the constitutive androstane (CAR) receptors. These are well known also as xenobiotic sensors, which are activated by numerous compounds leading to the activation of their downstream target genes (Xie, 2009).

The binding of xenobiotics to the receptor directly affects the clearance of those compounds, and consequently, protects the body from foreign

chemicals. Many drug metabolising enzymes involved in the metabolism of endogenous cellular regulators (steroids, eicosanoids) can be induced by hormones. For example, the growth hormone has been also proven to alter CYP expression (Mode et al., 1992, Cheung et al., 2006).

Inhibitor-mediated interaction involves a stabilisation mechanism, i.e. the drug decreases the degradation of CYP and therefore the concentration of the enzyme is increased (Watkins et al., 1986).

Phase II metabolising enzymes such as UGTs and GSTs can also be inducible. Induction of UGTs is highly dependent on the nature of the inducer. Ethanol and phenobarbital are able to induce UGTs, as well as the endogenous compound bilirubin (Soars et al., 2004, Hiroshi and Shigeko Shinkai and Takaharu, 2008). GSTs can also be induced by phenobarbital and other xenobiotics such as dithiolethiones (Zhang and Munday, 2008, Sasaki et al., 1989).

1.4.2 Inhibition of drug metabolising enzymes

In contrast to the effects observed in the induction of drug metabolic enzymes, inhibition delays xenobiotic biotransformation resulting in a higher concentration of the compound in the bloodstream. The consequence of such inhibition is the increased adverse reactions due to exacerbated pharmacological and toxicological effects (Kobayashi et al., 2002).

An inhibitor is a substance that interferes with the action of the enzyme, which will slow down the speed of the reaction. A reversible inhibitor will bind to an enzyme and will subsequently be released, whereas an irreversible inhibitor reacts with the enzyme producing a protein that will not be enzymatically active anymore and the original enzyme cannot be regenerated (Ring et al., 2014).

The two main types of CYP inhibition are called competitive and non-competitive inhibition. The former happens when an inhibitor competes with the substrate to bind to the active site of the enzyme. In this case, the inhibitor will block the access of the substrate to the enzyme. On the other hand, in the case of a non-competitive inhibition, both substrate and inhibitor will bind to different

sites of the enzyme. However, the enzyme cannot catalyse the reaction in as an efficient manner as it can in the absence of the inhibitor (Campbell and Farrell, 2012). Drugs like indinavir and saquinavir are competitive inhibitors, whereas ketoconazole and fluconazole can act as non-competitive inhibitors of CYP3A (Thummel and Wilkinson, 1998).

Like phase I enzymes, phase II enzymes can also display inhibitory activity. A study recently performed by Liu and coworkers (2016) demonstrated that UGT isoforms can be inhibited by vitamin A (Liu et al., 2016), whereas Chang and coworkers (1998) showed that oestrogens are able to inhibit GSTs (Chang et al., 1998).

1.5 FACTORS THAT INFLUENCE DRUG BIOTRANSFORMATION

In addition to the induction and inhibition of drug metabolising enzymes, several factors can contribute to the variability in biotransformation. They include:

- Genetic polymorphism
- Disease
- Age
- Sex
- Environmental factors

1.5.1 Genetic Polymorphism

Responses to drug consumption differ between individuals due to the variability of CYP content. A predefined dosage of a medicine might be enough to exert a pharmacological effect in one patient while needing to be adjusted to achieve the same effect in another patient. This can be explained by genetic polymorphisms within CYPs that can affect the metabolism of xenobiotics in general, leading to changes in drug responses and increased risk of ADRs (Zhou et al., 2009). For example, the bioavailability of omeprazole, which is a drug that is metabolised by both CYP3A4 and CYP2C19, has been demonstrated to be slower in Mexicans when compared with Caucasians but faster than that of Asians. The ethnic mixture of the Mexican population is a

plausible explanation for differences in the metabolic response compared to other ethnic groups (Gonzalez et al., 2003).

The genomic diversity of the Brazilian population is a result of the genetic admixture of three groups: Europeans, Africans, and Amerindians. Due to this very distinctive miscegenation, polymorphisms in CYP levels are undoubtedly present among Brazilians (Suarez-Kurtz, 2005). The Brazilian National Pharmacogenetics/Pharmacogenomics Network (REFARGEN) carried out studies in four regions of Brazil in order to map the genetic diversity of the population (Suarez-Kurtz, 2004). The study divided the population into three distinct races: white (mainly of Europeans ascendancy), brown (indigenous population) and black (mainly of African descent). The results obtained from REFARGEN showed that numerous polymorphisms were found on cytochromes 1A2, 2C9, 2C19, 2D6, and 3A5 (Figure 1-7).

Although the REFARGEN study attempted to map the genetic polymorphisms among the admixture races in Brazil we cannot affirm that there will be a correlation between the genetic polymorphisms observed in a group of brown Brazilian living in the north to brown Brazilians living in the south of the country for example.

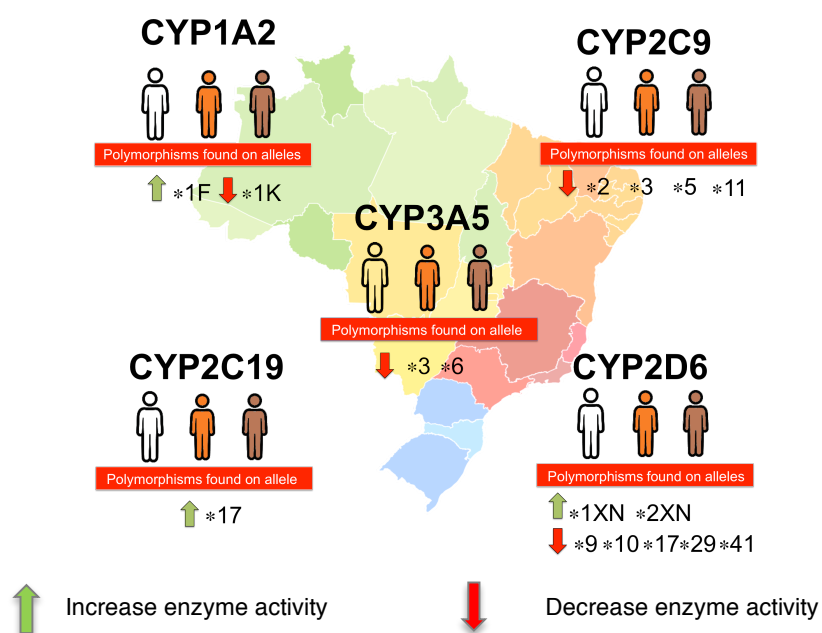


Figure 1- 7 Genetic polymorphisms found on CYP1A2, 2C9, 2C19, 2D6, and 3A5 among the Brazilian population

1.5.2 Disease

Diseases affecting the liver, such as cirrhosis, alcoholic liver disease, cholestatic jaundice, and liver carcinoma can alter organ function, and consequently, reduce the capacity of the body to clear drugs. Drug metabolism can also be affected by endocrine disorders such as diabetes mellitus, hypo- and hyperthyroidism, pituitary disorders and bacterial and viral infections in general. For example, in the case of a hepatic cirrhosis, the CYP2A6 can be overexpressed. This isoform catalyses the bioactivation of many drugs and also carcinogens. Therefore, CYP2A6 is a major liver catalyst in pathological conditions (Ionescu and Caira, 2005).

Malaria, which is one of the most common tropical diseases, can also affect the enzymes involved in the biotransformation of drugs. An impairment of microsomal drug metabolising activities as a result of the malaria infection reduces the oxidation of drugs by decreasing hepatic CYP activity (Mansor et al., 1991).

1.5.3 Age

Biotransformation contrasts can be found between newborns, the young and elderly due to the types and the amount of enzymes that are activated and expressed among those groups. Newborns are more sensitive to drug reactions because of the low level of development of their metabolising capacity. The sensitiveness of newborns lowers significantly over time until they reach adulthood when normal levels of enzyme activity are reached. In the elderly population, the biotransformation capacity may be reduced up to 30% due to several reasons. Reasons include decreased hepatic blood flow and mass, decreased absorption surface and gastrointestinal mobility and increased gastric pH (Klotz, 2009).

1.5.4 Sex

Sex is another factor that contributes to differences in the expression of drug metabolising enzymes. Initial findings on this subject were made in the 1930s when researchers treated rats with barbiture and observed that the

female ones required only half the dose to induce sleep compared to male rats (Gibson and Skett, 1986).

Another study discovered that the growth hormone may be the main hormonal factor that dictates the differences in the expression of CYPs and other drug metabolising enzymes between males and females (Waxman and Holloway, 2009).

1.5.5 Environmental factors

Heavy metals, industrial pollutants, pesticides and other chemical substances that are spread out in the air can potentially alter the activity of CYP enzymes in liver microsomes (Gillette, 1976). The activity of some drug metabolising enzymes may be induced if the individual is exposed to exogenous chemicals, including environmental pollutants. For example, most industrial pollutants are typically aromatic or aromatic polycyclic compounds and polychlorinated biophenols, which are known to cause inductive enzyme effects in various CYP isoforms (O'Mahony and Woodhouse, 1994, Kietz and Fischer, 2003).

1.6 HERB-DRUG INTERACTIONS AND ADVERSE REACTIONS

As previously discussed in section 1.2, reports on HDI are dated back to 1967 and since then over 1,500 articles have been published according to PubMed. The documented interactions involve well-known herbs from Traditional Chinese Medicine (TCM), Ayurveda and many other traditional herbal medicines commonly used by the population of many countries and their biotransformation involves many metabolic and transporter mechanisms.

1.6.1 Herb-drug interactions involving cytochrome P450

In section 1.3.1 we showed that the CYP family is responsible for the metabolism of most xenobiotics. Among the various CYP isoforms, CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A(4/5/7) are the most relevant ones because altogether they are responsible for the biotransformation of the majority of the currently marketed drugs (Mazzari and Prieto, 2014a).

Due to the increased popularity of SJW as an antidepressant, numerous interactions with CYP3A4 have been detected *in vitro* and *in vivo*, the interaction with oral contraceptives being one of the most remarkable cases of HDI (Murphy et al., 2005). As the interaction with CYP3A4 became more evident among researchers, the effects of SJW in other metabolic targets, such as CYP1A2, 2C9, 2C19, 2D6, 2E1 were also assessed. The numerous publications alerting to the potential interactions of SJW involving CYPs due to enzyme induction and/or inhibition made it possible to discuss the potential effects that other herbal medicines could cause on drug metabolising enzymes.

Besides SJW, popular herbal medicines such as *Ginkgo biloba* L. and milk thistle (*Silybum marianum* (L.) Gaertn. - *Compositae*) have been extensively studied over the past years in order to reveal their potential to cause HDI due to their effects on CYP activity and/or expression. Published studies showed that *Ginkgo biloba* and milk thistle are able to modulate the effect of the main CYP isoforms due to the inducible and/or inhibitory effects of their constituents (von Moltke et al., 2004, Venkataramanan et al., 2000).

A study published by Sugiyama and coworkers (2004) showed that simultaneous administration of *Ginkgo biloba* extract with the oral anti-diabetic tolbutamide potentiated the hypoglycaemic action of this drug in rats. Tolbutamide is metabolised by CYP2C9 and the inhibitory effect caused by the *Ginkgo biloba* extract increased both the plasma concentrations and the bioavailability of the drug. Hence, blood glucose levels dramatically dropped in rats treated with the drugs (Sugiyama et al., 2004).

Silybin, a constituent of milk thistle, show inhibitory effects on CYP2D6, 2E1, and 3A4 (Zuber et al., 2002). The ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 from *Panax ginseng* C.A.Mey. (*Araliaceae*) display inductive and inhibitory effects on CYP 1A2, 2C9, 2C19, 2D6, and 3A4 (Henderson et al., 1999). A drug interaction study demonstrated that silymarin (140mg) given three times a day inhibited hepatic clearance of losartan, which is a drug used to treat high blood pressure, in Chinese subjects. The active metabolite of losartan (E-3174) is formed by CYP2C9. Due to the inhibition of the enzyme caused by the milk thistle constituent silymarin, the amount of E-3174 found in

the subjects treated also with the herbal medicine decreased compared to the control group (Brantley et al., 2014).

A plethora of data on HDI involving CYP has been published demonstrating that herbal medicines are able to affect CYP and therefore their consumption should be avoided with a synthetic drug. Nonetheless, evaluation of this subject is very controversial. The fact that most of the published research on HDI is either *in vitro*, performed in animal models and done with fractions (not whole herbs) at non-physiological concentrations, explains why most of the 'theoretical' HDI have never been clinically observed. Preclinical data is very important because it serves as a guide for clinical studies. Therefore, it is important that such studies are fully performed and are not abandoned in their initial or middle stages, which has been the case in much research on herbal medicines that has never reached the clinical trials stage of development (Brantley et al., 2014).

1.6.2 Herb-drug interactions involving phase II metabolising enzymes

Glucuronidation is a major route of phase II xenobiotic biotransformation and elimination in the body. Classes of drugs such as narcotic analgesics, anxiolytics, and NSAIDs are examples of therapeutic agents metabolised via glucuronidation mechanism. Due to the high number of exogenous compounds that are metabolised through this route, the characterisation of the effects of herbal medicines on the activity of UGTs is very important to reveal potential HDI (Ionescu and Caira, 2005).

In vitro studies demonstrated that valerian extract (*Valeriana officinalis* L. - *Caprifoliaceae*) - used at clinical dosages are able to produce significant reductions in the glucuronidation of conventional drugs like acetaminophen and morphine, possibly caused by the inhibition of several UGTs such as UGT1A6/9, UGT1A1, and UGT2B7 (Alkharfy and Frye, 2007).

Echinacea, ginkgo, milk thistle, soy, and SJW are other extracts that have been assessed for their effects on UGT *in vitro*. The extracts display inhibitory effects on UGT1A1, UGT 1A9, and UGT2B15. Constituents of several herbal medicines, such as silymarin and silybin (milk thistle), genistein (soy),

hypericin (SJW) and valerianic acid (valerian) are also able to modulate glucuronidation due to the inhibition and/or induction of various UGTs isoforms (Mohamed and Frye, 2011).

GSH conjugation is also affected by xenobiotics due to the modulatory effects on GSTs. A study conducted by Yang and coworkers (2003) showed that *Ginkgo biloba* extract down-regulates the GST activity in microsomes from rat kidney (Yang et al., 2003). Lower GST activity can lead to potential adverse reactions caused by toxic metabolites that are detoxified by conjugation with GSH such as N-acetyl-p-benzoquinoneimine (NAPQI) (Henderson et al., 2000). GSH conjugation is the detoxification mechanism that various drugs undergo, including anticancer agents. Even though elevated levels of GSH are considered positive due to its strong antioxidant effects in the cells, the high activity of GSTs can weaken the effectiveness of certain anticancer drugs. GST P1-1, for example, is particularly induced in certain types of cancer, such as lung, colon, and stomach cancers. Because of its increased activity, the GST P1-1 enzyme is implicated in cellular resistance to chemotherapeutic agents (Ramsay and Dilda, 2014).

Many other HDI involving phase II metabolising enzymes have been reported in the literature. As some conjugation mechanisms are the major detoxifying route of several important classes of drugs, such as the anticancer agents, more interaction studies (especially clinical studies) need to be carried out on herbal medicines that could potentially affect phase II enzymes and reactions.

1.6.3 Herb-drug interactions involving P-glycoprotein

Modulation of drug transporters like P-gp by herbal medicines has been the target of a plethora of studies. Due to its high expression and activity in several tissues (kidney, liver, intestine, and blood-brain barrier (BBB)), which are pharmacologically important epithelial barriers, the bioavailability of the drug could be compromised if P-gp activity is affected (Li et al., 2014).

Inhibition or induction of P-gp activity results in altered absorption and bioavailability of P-gp substrates (Zhou et al., 2004). Studies have shown that

polyphenols from green tea (*Camellia sinensis* (L.) Kuntze – Theaceae) are able to inhibit P-gp activity, which is bolstered by the intracellular accumulation of the P-gp substrate rhodamine 123 (Rh-123) in Caco-2 cells (Jodoin et al., 2002).

SJW displays inductive effects in P-gp efflux activity. Chronic treatment with SJW increases the P-gp efflux in healthy subjects, which can also be observed *in vitro* by a reduced intracellular accumulation of the P-gp substrate Rh-123 (Hennessy et al., 2002). This inductive effect on P-gp has been demonstrated to attenuate the efficacy of the potent P-gp inhibitor drug ritonavir, which potentially leads to treatment failure (Drewe et al., 1999).

Ezuruike and Prieto (2014) showed that some herbal medicines used in Nigeria for diabetes management can cause HDI at P-gp level. This is the case of the coadministration of medicinal plants such as *Acacia nilotica* (L.) Delile (Leguminosae), *Annona senegalensis* Pers. (Annonaceae), *Bauhinia thonningii* Schum. (Leguminosae), *Bridelia ferruginea* Benth. (Phyllanthaceae), *Carica papaya* L. (Caricaceae) and *Morinda lucida* Benth. (Rubiaceae) with the antidiabetic drug glibenclamide. According to the authors, the herbal medicines which exhibit inhibitory effects on P-gp and their concomitant use with glibenclamide increases the plasma concentration of the synthetic drug (Ezuruike and Prieto, 2014).

Some examples of potential HDI previously mentioned involving metabolising enzymes and P-gp were conducted using *in vitro* and *in vivo* models in order to either predict or attest an interaction that has been already reported by other researchers. However, HDI of the most of the marketed herbal medicines have not been evaluated using the usual methods of clinical pharmacology, that is, preclinical metabolic studies have not been conducted (Skalli and Soulaymani Bencheikh, 2012). Therefore, pharmacovigilance plays a very important role in the detection of HDI. Unfortunately, in many cases, an alert about possible HDI is only made after a reported case of death believed to be due to treatment failure or increased toxicity of a synthetic drug.

1.7 PHARMACOVIGILANCE OF HERBAL MEDICINES

The pharmacological and PK metabolic knowledge on herbal drugs accumulated over the last decades (summarised in the previous sections) prompted WHO to make an alert as to the potential risks of uncontrolled use of herbal medicines in conjunction with other synthetic drugs. In 2004, WHO issued the “Guidelines on safety monitoring of herbal medicines in pharmacovigilance systems”. These guidelines indicate how member countries should include herbal medicines within existent pharmacovigilance systems in order to facilitate the exchange of information on adverse reactions and HDI between WHO member countries (WHO, 2004).

The WHO traditional medicine strategy, which was published by WHO in 2013, showed that the regulatory status of herbal medicines is quite diverse among WHO member countries. A medicinal plant can be registered as a food, a functional food, a dietary supplement, a traditional medicine, or a full licensed herbal medicine depending on the country’s own needs, cultural background, history of use of medicines, and regulations. This can potentially cause difficulties to pharmacovigilance systems in collecting HDI data, as for example a food interaction would be practically ignored and hardly detected by health professionals (WHO, 2013, SUS, 2006).

In addition to the differences in the regulatory status of herbal medicines between many countries, other challenges can be highlighted in terms of pharmacovigilance of herbal medicines. The presence of numerous active compounds in herbal extracts, the low standardisation of herbal products, the problems with the nomenclature of herbs and the many sources of the herbal medicines used by the patients are also examples that make the detection of HDI by health professionals more difficult. As a consequence, HDI are likely to be underreported to the pharmacovigilance systems (Shaw et al., 2012).

1.8 USE OF HERBAL MEDICINES IN BRAZIL AND THE RISK OF HERB- DRUG INTERACTIONS ON PATIENTS OF THE PUBLIC HEALTH SYSTEM (SUS)

Brazil is the most biodiverse country on the planet and a valuable traditional knowledge associated with the use of medicinal plants. The total biodiversity of the country corresponds to between 15 and 20% of the plant species that are found in the world, which also encompasses medicinal plants. Although the country has about 60,000 catalogued plant species, only 8% have been studied for their bioactive compounds and 1,100 have been assessed for their medicinal properties (Dutra et al., 2016). The vast genetic heritage found in the country gives to Brazil the opportunity to be a leading country in natural products research that could potentially benefit millions of people who still have no access to costly treatments with synthetic drugs and are affected by neglected tropical conditions, such as dengue fever, chikungunya and the most recent threat, the zika fever (SUS, 2006).

Agriculture in Brazil is the main source of income for millions of families in the country. Over the years, the Brazilian government has made expressive investments in this field in order to develop this government sector and to improve the research into the cultivation of plants (especially medicinal plants) (SUS, 2006).

Therefore, Ministry of Agriculture and Ministry of Health have stimulated the development of the medicinal plants field in Brazil. They aim to integrate the traditional knowledge of both cultivation and medicinal use of Brazil's biodiversity in order to offer alternative treatments to the entire population of the country by increasing the availability of herbal medicines in the Brazilian Unified Health System (SUS) (SUS, 2006).

The SUS was created by the Brazilian Federal Constitution of 1988. According to the document, SUS has to guarantee full and free access to various health procedures and treatments for the entire population of Brazil. Currently, 180 million people rely exclusively on the health services provided by SUS. All medications available at SUS have been put together in a list of

essential medicines called RENAME. The list was created in 1975, before the creation of the SUS, and is based on the WHO recommendations to the member countries to develop a list of medicines aimed to attend to the health needs of the population. Since then the list has been revised and updated according to newly available treatments for the patients of SUS. At the moment, RENAME contains 440 drugs that are prescribed to treat most of the conditions that affect the Brazilian population (Brasil, 2014).

Between 2013 and 2015, the search for treatments using herbal medicines in SUS grew 161% and over the last 3 years at least 6,000 Brazilians went to local pharmacies seeking the use herbal drugs (Brasil, 2016). Currently, only 12 herbal medicines are listed in RENAME, therefore limiting the prescription of those drugs to the patients of SUS (Figueiredo et al., 2014).

In 2008, the Ministry of Health of Brazil issued a list of medicinal plants of interest of SUS (RENISUS). This list contains medicinal plants that have the potential to generate products for SUS (Table 1-2). As part of the selection process of the medicinal plants for the list, the Ministry of Health coordinated regular meetings with Brazilian researchers, representatives of the Brazilian Pharmacopoeia (BP) and the National Health Surveillance Agency (ANVISA). The work started in 2005 with a list of 237 plant species. The preliminary list considered plant species that were already in use in health services of the states and municipalities, the traditional and popular knowledge, the available chemical and pharmacological studies, and other plants that were in use by other health programs already established throughout the country. After several meetings, RENISUS was created containing various medicinal plant species that, eventually, should be available for the patients of SUS as part of RENAME (Brasil, 2009).

Some of RENISUS species are widely used worldwide, for example *Cynara scolymus* L. (Compositae), *Allium sativum* L. (Amaryllidaceae) and *Uncaria tomentosa* (Wild. Ex Schult.) DC. (Rubiaceae). Numerous medicinal plants that are traditionally used in the country are also present in the BP. Monographs of medicinal plant species have been included in the BP since the launch of its first edition in 1926. From this date, four more editions have been

published, dating: 1959, 1977, 1996, and 2010 with a special edition issued in 2011. More than 1000 monographs of medicinal plants, including native and non-native plants to Brazil, have been included in the editions (Brandão et al., 2006, ANVISA, 2011).

More than 500 herbal medicines were registered at ANVISA by 2008 and this number has certainly increased as the number of users grew as well. *Ginkgo biloba* L., *Aesculus hippocastanum* L. (Sapindaceae), *Cynara scolymus* L., *Hypericum perforatum* L., *Glycine max* (L.) Merr. (Leguminosae), *Valeriana officinalis* L., *Panax ginseng* C.A.Mey., *Cassia angustifolia* M.Vahl. (Leguminosae), *Cimicifuga racemosa* (L.) Nutt. (Ranunculaceae), *Mikania glomerata* Spreng., *Maytenus ilicifolia* Mart. ex Reissek (Celastraceae) and *Peumus boldus* Molina (Monimiaceae) are listed as the most used medicinal plant species by the Brazilian population. The native plants *Mikania glomerata* Spreng., *Maytenus ilicifolia* Mart. ex Reissek and *Paullinia cupana* Kunth (Sapindaceae) are widely used in the country as a bronchodilator, adjuvant in the treatment of gastric ulcers and as a stimulator of the nervous system respectively (Carvalho et al., 2008).

Due to the high number of synthetic drugs available in SUS, cases of interactions are likely to occur when the patient has been prescribed a treatment with more than one drug. As a consequence, the number of drug interactions (especially cases of HDI at the absorption and metabolic levels) may increase with the implementation of RENISUS. Thus, studies on the effects of those herbal medicines in metabolic and transporter mechanisms are highly required in order to anticipate HDI that could affect the patients of SUS.

Table 1- 2 RENISUS plant species, traditional uses and presence in the Brazilian pharmacopoeia.

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Achillea millefolium</i> L. (Compositae)	√ (ANVISA, 2011)	Antipyretic, anti-inflammatory, haemostatic, spasmolytic, diuretic, diaphoretic, hypotensive.	(Williamson and Wren, 2003)
<i>Allium sativum</i> L. (Amaryllidaceae)	√ (Brandão et al., 2006)	Antibiotic, expectorant, hypotensive, antithrombotic, anti-diabetic.	(Williamson and Wren, 2003)
<i>Aloe barbadensis</i> Mill. = <i>Aloe vera</i> (L.) Burm.f. (Xanthorrhoeaceae)	√ (Brandão et al., 2006)	Purgative, emmenagogue, aid wound healing, relieve burns, colonic irrigation, anti-inflammatory, anti-ulcerogenic, anti-tumor, immunomodulatory.	(Williamson and Wren, 2003)
<i>Alpinia zerumbet</i> (Pers.) B.L.Burt & R.M.Sm (Zingiberaceae)	—	For treatment of intestinal and cardiovascular diseases.	(Leal-Cardoso et al., 2004)

RENISUS plant species and family	Brazilian Pharmacopoeia	Some traditional uses	Reference
<i>Alpinia speciosa</i> (Blume) D.Dietr. = <i>Etlingera elatior</i> (Jack) R.M.Sm. (Zingiberaceae)	—	For treatment of earache, anti-tumor promoting and for healing wounds.	(Chan et al., 2011, Abdelwahab et al., 2010)
<i>Anacardium occidentale</i> L. (Anacardiaceae)	√ (Brandão et al., 2006)	For treatment of toothache, sore gums and febrifuge in malaria.	(Williamson and Wren, 2003)
<i>Ananas comosus</i> (L.) Merr. (Bromeliaceae)	—	Anti-inflammatory, anti-oedematous, anti-arthritic, analgesic and wound-healing.	(Williamson and Wren, 2003)
<i>Apuleia ferrea</i> (Mart.) Baill. = <i>Caesalpinia ferrea</i> Mart. (Leguminosae)	—	For treatment of gastroduodenal diseases.	(Bacchi et al., 1995)
<i>Arrabidaea chica</i> (Bonpl.) Verl. = <i>Fridericia chica</i> (Bonpl.) L.G.Lohmann (Bignoniaceae)	—	For treatment of anaemia, haemorrhage, inflammation, intestinal colic, hepatitis, leukaemia and skin infections.	(Gemelli et al., 2015, Michel et al., 2015)
<i>Artemisia absinthium</i> L. (Compositae)	√ (Brandão et al., 2006)	Choleretic, anthelmintic, stomachic and anti-inflammatory.	(Williamson and Wren, 2003)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Baccharis trimera</i> (Less.) DC. (Compositae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Anti-diabetic.	(Oliveira et al., 2005)
<i>Bauhinia affinis</i> Vogel = <i>Bauhinia aculeata</i> L. (Leguminosae)	—	Anti-diabetic.	(Gomez-Estrada et al., 2011)
<i>Bauhinia forficata</i> Link. (Leguminosae)	—	Anti-diabetic.	(Damasceno et al., 2004)
<i>Bauhinia variegata</i> L. (Leguminosae)	—	Antileprotic, antioitrogenic, antibacterial, antitumor, dysmenorrhea, tonic, astringent, for treatment of menorrhagia, tuberculosis, asthma, liver tonic, edema suppression, dysentery, ulcers, eye disease, skin diseases, fever and haemorrhoids.	(Pani et al., 2011, Mali and Dhake, 2011)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Bidens pilosa</i> L. (Compositae)	—	Anti-cancer, anti-inflammatory, anti-diabetic, anti-hyperglycemic, antioxidant, immunomodulatory, antimalarial, antibacterial, antifungal, antihypertensive, vasodilatory and antiulcerative.	(Bartolome et al., 2013)
<i>Calendula officinalis</i> L. (Compositae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Anti-inflammatory, spasmolytic, anti-haemorrhagic, styptic, vulnerary, antiseptic.	(Williamson and Wren, 2003)
<i>Carapa guianensis</i> Aubl. (Meliaceae)	—	Anti-inflammatory, anti-allergic, skin diseases, arthritis, rheumatism, infections, wounds, bruises and analgesic.	(Henriques and Penido, 2014, Brito et al., 2013)
<i>Casearia sylvestris</i> Sw. (Salicaceae)	√ (ANVISA, 2011)	Anti-diarrheic, anti-ophidian, flu and chest colds, treatment of gastric ulcers, wounds, burns, rashes, eczema, vitiligo and herpes.	(Ferreira et al., 2011, Esteves et al., 2005)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Chamomilla recutita</i> (L.) Rauchert = <i>Matricaria chamomilla</i> L. = M. recutita L (Compositae)	—	Sedative, carminative, antispasmodic, analgesic, anti-inflammatory and antiseptic.	(Williamson and Wren, 2003)
<i>Chenopodium ambrosioides</i> L. = <i>Dysphania ambrosioides</i> (L.) Mosyakin & Clemants (Amaranthaceae)	√ (Brandão et al., 2006)	Anthelmintic.	(Williamson and Wren, 2003)
<i>Copaifera sp*</i>	—	* Species to be used have not yet been defined by the Ministry of Health.	—
<i>Cordia verbenacea</i> DC. = <i>Cordia curassavica</i> (Jacq.) Roem. & Schult. (Boraginaceae)	√ (ANVISA, 2011)	Antibacterial, anti-inflammatory, analgesic and antifungal.	(Rodrigues et al., 2012, Matias et al., 2013)
<i>Costus scaber</i> Ruiz & Pav. (Costaceae)	—	Antibacterial, febrifuge, nephritic pain and antigonorrhoeic.	(Lans et al., 2001, Breitbach et al., 2013)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Costus spicatus</i> (Jacq.) Sw. (Costaceae)	—	Anti-inflammatory.	(Quintans Junior et al., 2010)
<i>Croton cajucara</i> Benth. (Euphorbiaceae)	—	For treatment of gastrointestinal disorders.	(do Socorro et al., 2003)
<i>Croton zehntneri</i> Pax. & K. Hoffm. (Euphorbiaceae)	—	Antispasmodic, cardiovascular and gastroprotective.	(da Silva-Alves et al., 2015, Batatinha et al., 1995)
<i>Curcuma longa</i> L. (Zingiberaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Anti-inflammatory, anti-hepatotoxic, digestive, blood purifier, antiseptic and general tonic.	(Williamson and Wren, 2003)
<i>Cynara scolymus</i> L. (Compositae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Choleretic, digestive, anti-emetic, liver protectant, hypolipidaemic, antispasmodic and antioxidant.	(Williamson and Wren, 2003)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Dalbergia subcymosa</i> Ducke (Leguminosae)	—	Anti-inflammatory, antimicrobial.	(Peters and Guerra Mde, 1995, Oliveira et al., 2013)
<i>Eleutherine plicata</i> (Sw.) Herb. = <i>Eleutherine bulbosa</i> (Mill. Urb. (Iridaceae)	—	For treatment of giardiasis, amoebiasis and for treatment of diarrhoea.	(Couto et al., 2016)
<i>Equisetum arvense</i> L. (Equisetaceae)	—	Haemostatic, astringent, cystitis, prostatitis, urethritis, enuresis, anti-haemorrhagic.	(Williamson and Wren, 2003)
<i>Erythrina mulungu</i> Benth. = <i>Erythrina verna</i> Vell. (Leguminosae)	√ (Brandão et al., 2006)	For treatment of insomnia and depression.	(Flausino et al., 2007b, Flausino et al., 2007a)
<i>Eucalyptus globulus</i> Labill. (Myrtaceae)	√ (Brandão et al., 2006)	Antiseptic, antispasmodic, expectorant, stimulant, febrifuge, insect repellent.	(Williamson and Wren, 2003)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Eugenia uniflora</i> L. = <i>Myrtus brasiliiana</i> L. (Myrtaceae)	√ (Brandão et al., 2006)	Anti-diarrheic, diuretic, antirheumatic, antihypertensive and antifebrile.	(Schapoval et al., 1994, Consolini et al., 1999)
<i>Foeniculum vulgare</i> Mill. (Apiaceae)	√ (Brandão et al., 2006)	Stomachic, carminative, anti-inflammatory, orexigenic.	(Williamson and Wren, 2003)
<i>Glycine max</i> (L.) Merr. (Leguminosae)	—	Hormone-replacement-therapy.	(Williamson and Wren, 2003)
<i>Harpagophytum procumbens</i> DC. Ex (Pedaliaceae)	—	Anti-inflammatory, anti-rheumatic, analgesic, sedative.	(Williamson and Wren, 2003)
<i>Jatropha gossypifolia</i> L. (Euphorbiaceae)	—	Antihypertensive, anti-inflammatory, antiophidian, analgesic, antipyretic, antimicrobial, healing, antianemic, antidiabetic, and antihemorrhagic.	(Felix-Silva et al., 2014)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Justicia pectoralis</i> Jacq. (Acanthaceae)	√ (ANVISA, 2011)	Anti-inflammatory, antimicrobial, bronchodilator and anxiolytic.	(Venancio et al., 2011)
<i>Kalanchoe pinnata</i> (Lam.) Pers. = <i>Bryophyllum pinnatum</i> (Lam.) Oken (Crassulaceae)	—	Anti-inflammatory, antileishmanial.	(Torres-Santos et al., 2003)
<i>Lamium album</i> L. (Lamiaceae)	—	Astringent and haemostatic.	(Williamson and Wren, 2003)
<i>Lippia sidoides</i> Cham. (Verbenaceae)	√ (ANVISA, 2011)	Antimicrobial, skin cuts, insect bites and sore throat.	(Botelho et al., 2007)
<i>Malva sylvestris</i> L. (Malvaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Demulcent, pectoral, for treatment of colds, coughs, insect stings and bites.	(Williamson and Wren, 2003)
<i>Maytenus aquifolium</i> Mart. = <i>Maytenus ilicifolia</i> Mart. ex Reissek (Celastraceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Antiulcer.	(Kimura et al., 2000, Corsino et al., 2003)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Mentha crispa</i> L. = <i>Mentha spicata</i> L. (Lamiaceae)	—	Carminative and for treatment of giardiasis, amebiasis and urogenital Trichomoniasis.	(Teles et al., 2011)
<i>Mentha x piperita</i> L. (Lamiaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Spasmolytic, carminative, antiemetic, diaphoretic and antiseptic.	(Williamson and Wren, 2003)
<i>Mentha pulegium</i> L. (Lamiaceae)	—	Carminative, diaphoretic, stimulant and emmenagogue.	(Williamson and Wren, 2003)
<i>Mentha x villosa</i> Huds. (Lamiaceae)	—	Anxiolytic and for treatment of stomachache, menstrual cramps and diarrhoea.	(Sousa et al., 2009)
<i>Mikania glomerata</i> Spreng. (Asteraceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Bronchodilator, antiallergic and antiasthmatic.	(Santana et al., 2014)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Mikania laevigata</i> Sch. Bip. ex Baker (Compositae)	√ (ANVISA, 2011)	For treatment of inflammatory conditions, bronchodilator activity, anti-ulcerogenic, antiophidian as well as antibacterial and antiparasitic activity.	(Napimoga and Yatsuda, 2010)
<i>Momordica charantia</i> L. (Cucurbitaceae)	—	For treatment of diabetes, asthma, skin infections and hypertension.	(Williamson and Wren, 2003)
<i>Morus nigra</i> L. (Moraceae)	√ (Brandão et al., 2006)	Laxative, diuretic, hypotensive, expectorant and reduction of blood sugar levels.	(Williamson and Wren, 2003)
<i>Ocimum gratissimum</i> L. (Lamiaceae)	—	Antibacterial, antidiabetic and for treatment of many ailments, including upper respiratory tract infections, diarrhoea, headache, fever, eye problems, skin diseases, and pneumonia.	(Ofem et al., 2012)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Orbignya speciosa</i> Barb. Rodr. = <i>Attalea speciosa</i> Mart. (Arecaceae)	—	Anti-inflammatory.	(de Souza et al., 2011)
<i>Passiflora edulis</i> Sims. = <i>Passiflora incarnata</i> L. (Passifloraceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Stimulant, antispasmodic, anti-inflammatory, antianxiety, anti-tumor, antimicrobial, antioxidant, tonic, hypotensive, anodyne, anticarcinogenic, sedative, hypnotic and for treatment of insomnia and gastrointestinal complaints.	(Devaki et al., 2012, Williamson and Wren, 2003)
<i>Passiflora alata</i> Curtis. (Passifloraceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Anxiolytic, sedative, diuretic and analgesic.	(Barbosa et al., 2008, Rudnicki et al., 2007)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Persea americana</i> Mill. = <i>Persea gratissima</i> C.F.Gaertn. (Lauraceae)	√ (Brandão et al., 2006)	Abortifacient, emmenagogue, contraceptive, anti-diarrhoeal, anti-hypertensive and diuretic.	(Williamson and Wren, 2003)
<i>Petroselinum sativum</i> Hoffm. = <i>Petroselinum crispum</i> (Mill.) Fuss (Apiaceae)	√ (Brandão et al., 2006)	Diuretic, spasmolytic, carminative, aperient, antiseptic, expectorant, antirheumatic and sedative.	(Williamson and Wren, 2003)
<i>Phyllanthus amarus</i> Schumach. & Thonn. = <i>Phyllanthus niruri</i> L. (Phyllanthaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Diuretic, liver protectant and tonic, choleric, anti-inflammatory, kidney conditions and gallstones.	(Williamson and Wren, 2003)
<i>Phyllanthus tenellus</i> Roxb. (Phyllanthaceae)	√ (Brandão et al., 2006)	For treatment of kidney and bladder calculi, diabetes, hepatitis, jaundice and asthma.	(Ignacio et al., 2001)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Phyllanthus urinaria</i> L. (Phyllanthaceae)	—	Anti-inflammatory, antiviral, antibacterial, anticancer and antihepatotoxic.	(Huang et al., 2014)
<i>Plantago major</i> L. (Plantaginaceae)	√ (ANVISA, 2011)	Anti-inflammatory, analgesic, diuretic and antihemorrhagic.	(Williamson and Wren, 2003)
<i>Plectranthus barbatus</i> Andrews = <i>Coleus barbatus</i> (Andrews) Benth. ex G.Don (Lamiaceae)	√ (ANVISA, 2011)	For treatment of intestinal disturbance and liver fatigue, respiratory disorders, heart diseases and certain central nervous system disorders.	(Alasbahi and Melzig, 2010, de Almeida Alves et al., 2001)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Polygonum acre</i> Kunth = <i>Persicaria punctata</i> (Elliott) Small (Polygonaceae)	√ (ANVISA, 2011)	For treatment of haemorrhoids and rheumatism, diuretic and emenagogue.	(de Almeida Alves et al., 2001)
<i>Polygonum hydropiperoides</i> Michx. = <i>Persicaria hydropiperoides</i> (Michx.) Small (Polygonaceae)	—	Stimulant, diuretic and emmenagogue.	(Williamson and Wren, 2003)
<i>Portulaca pilosa</i> L. (Portulacaceae)	—	Analgesic, antipyretic, diuretic.	(Rocha et al., 1994)
<i>Psidium guajava</i> L. (Myrtaceae)	√ (Brandão et al., 2006)	Anti-inflammatory, antimicrobial, antioxidant, antidiarrheal, antimutagenic.	(Ravi and Divyashree, 2014)
<i>Punica granatum</i> L. (Lythraceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Taeniafuge and astringent.	(Williamson and Wren, 2003)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Rhamnus purshiana</i> DC. = <i>Frangula purshiana</i> Cooper (Rhamnaceae)	√ (Brandão et al., 2006)	Laxative.	(Williamson and Wren, 2003)
<i>Ruta graveolens</i> L. (Rutaceae)	√ (Brandão et al., 2006)	Antispasmodic and emmenagogue.	(Williamson and Wren, 2003)
<i>Salix alba</i> L. (Salicaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Analgesic, anti-inflammatory, febrifuge and tonic.	(Williamson and Wren, 2003)
<i>Schinus terebinthifolia</i> Raddi (Anacardiaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Anti-inflammatory and antimicrobial.	(Silva et al., 2010)
<i>Solanum paniculatum</i> L. (Solanaceae)	√ (Brandão et al., 2006)	For treatment of liver and gastrointestinal disorders and as an antimicrobial, anti-inflammatory, and antihypertensive.	(Mesia-Vela et al., 2002a, VIEIRA et al., 2013)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Solidago microglossa</i> DC. = <i>Solidago chilensis</i> Meyen (Compositae)	✓ (Brandão et al., 2006)	Astringent, and for treatment of bruises and stomach disorders.	(Bagatini et al., 2009)
<i>Stryphnodendron adstringens</i> (Mart.) Coville = <i>Stryphnodendron barbatimam</i> Mart. (Leguminosae)	✓ (Brandão et al., 2006)	For treatment of leucorrhoea, bleeding, diarrhoea, haemorrhoids and wounds.	(Souza et al., 2007)
<i>Syzygium jambolanum</i> (Lam.) DC = <i>Syzygium cumini</i> (L.) Skeels (Myrtaceae)	—	Anti-diabetic, astringent and diuretic.	(Williamson and Wren, 2003)
<i>Tabebuia avellanedae</i> Lorentz ex Griseb. = <i>Handroanthus impetiginosus</i> (Mart. ex DC.) Mattos (Bignoniaceae)	—	For treatment of infectious diseases including bacterial, fungal and viral infections, to enhance immune function and for cancer treatment.	(Williamson and Wren, 2003)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Tagetes minuta</i> L. (Compositae)	—	Anti-inflammatory, anti-spasmodic, anti-parasitic, anti-septic and sedative.	(Karimian et al., 2014)
<i>Trifolium pratense</i> L. (Leguminosae)	—	Alterative, antispasmodic, expectorant, sedative and dermatological agent.	(Williamson and Wren, 2003)
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC. (Rubiaceae)	—	For treatment of arthritis, rheumatism, asthma, gastric ulcer, skin diseases and cancer.	(Williamson and Wren, 2003)
<i>Vernonia condensata</i> Baker = <i>Acmella ciliata</i> (Kunth) Cass. (Compositae)	✓ (ANVISA, 2011)	Analgesic, anti-inflammatory, antipyretic, antianemic, antibacterial and antiulcerogenic.	(Boeing et al., 2016)

RENISUS plant species and family	Brazilian Pharmacopoeia	Selected traditional uses	Reference
<i>Vernonia polyanthes</i> Less. = <i>Vernonanthura phosphorica</i> (Vell.) H.Rob. (Compositae)	√ (ANVISA, 2011)	Antimalarial, antipyretic, hypotensive, and for treatment of bronchitis and persistent cough.	(Barbastefano et al., 2007)
<i>Zingiber officinale</i> Roscoe (Zingiberaceae)	√ (Brandão et al., 2006) √ (ANVISA, 2011)	Carminative, anti-emetic, spasmolytic, anti-flatulent, antitussive, hepatoprotective, anti-platelet and hypolipidaemic agent.	(Williamson and Wren, 2003)

—, Not present in the BP; √, Present in the BP

1.9 REPORTED EFFECTS OF RENISUS MEDICINAL PLANTS ON MAIN PHASE I AND PHASE II METABOLIC MECHANISMS AND THE P-GLYCOPROTEIN TRANSPORTER.

In this chapter, we showed that herbal medicines are able to modulate metabolic and transporter proteins and, as a consequence, cause HDI at both the absorption and metabolic levels if they are coadministered with synthetic drugs. Also, we highlighted that the number of users of herbal medicines in SUS has considerably increased over the years. Statistics indicate that about 66% of the Brazilian population have no access to commercial medicines (Trojan-Rodrigues et al., 2012). Even when access is granted, popular use of herbal medicines is often due to poor medical and pharmaceutical assistance and the high cost of treatment with conventional drugs (Silveira et al., 2008). Brazilians are also becoming more interested in “safe” and “natural” treatments aimed to promote healthier living. A consequence of this increased use of phytotherapy has been a higher number of case reports of adverse reactions caused by the uncontrolled consumption of herbal medicines (Silveira et al., 2008).

The ANVISA is in charge of the regulation of herbal medicines in the country and since its creation in 1999, many advances have been made in order to control and ensure their efficacy and safety (Balbino and Dias, 2010). In order to guarantee the safety of the consumption of phytomedicinal candidates by humans, ANVISA established a requirement that at least 20 years of prior traditional use must be attested. In the absence of this evidence the efficacy and safety of the candidate need to be demonstrated by a point based system according to literature data, clinical evidence or an indication that the herb is already included in the list of simplified registration of herbal medicines (ANVISA, 2010). Although these regulations are relevant to improve the safety and efficacy of herbal medicines, PK metabolic studies on the traditional medicinal plants are not yet a regulatory requirement. As a

consequence, there is a scarcity of this kind of data which is regarded as an oversight in terms of safety (Ribeiro et al., 2005).

As previously stated, the SUS is one of the biggest public health systems in the world and is responsible for the health care of 180 million Brazilian citizens (Mendes, 2013). In order to establish which medicines should be provided by SUS, the Brazilian Health Ministry produced a list of essential drugs (RENAME) according to the International Classification of Diseases (ICD) and epidemiological studies conducted nationwide (Brasil, 2014). Several herbal medicines are already part of this list, such as *Cynara scolymus* L., *Schinus terebinthifolia* Raddi and *Rhamnus purshiana* DC. However, due to the extensive number of plant species possessing pharmacological activity used in Brazil and the necessity for increased availability of herbal medicines in SUS, the Health Ministry determined that more herbal medicines should be provided by the System to the population, as part of one of the aims of the National Policy of Integrative and Complementary Practices (PNPIC). Consequently, RENISUS was published in 2008 and, from this point on, efforts have been concentrated on elucidating the efficacy and safety of the plant species present on the list (Brasil, 2009, Feijó et al., 2012).

The profile of pharmaceutical drugs at both the absorption and metabolic levels is essential to determine whether or not they will interact with other therapeutic interventions (Ionescu and Caira, 2005). However, studies on metabolic and transporter targets using plant extracts are very difficult to carry out because of their chemical complexity (Simões and Mariot, 2003). As a result, there is very little data for numerous native and exotic adapted medicinal plants that are traditionally used in Brazil (He et al., 2010). The *in vitro* profiles of some herbal medicines in metabolic and transporter mechanisms can be found in the literature and, although these data are important, they are seldom used for the prediction of potential HDI. Thus, we will provide an overview of the effects of medicinal plants to be used in the SUS in the main phase I and phase II metabolism targets and the P-gp transporter.

A literature search was conducted using the Library of Medicine's PubMed database between May and June 2013. Included studies consisted of

the reported effects of the Brazilian medicinal plants of RENISUS list on the main liver metabolic enzymes, which are involved in phase I (functionalization reactions mediated by CYP) and phase II (GSH conjugation, glucuronidation, sulphation, methylation and acetylation) metabolism and also the assessment of their effects on P-gp activity. The following combinations of keywords were used: 'Plant name AND, 1A2, 3A4, 3A5, 3A7, 2C9, 2C19, 2D6, 2E1', 'Plant name AND glutathione' or 'Plant name AND GSH', 'Plant name AND glucuronidation' or 'Plant name AND UGT', 'Plant name AND sulphation', 'Plant name AND sulphate conjugation', 'Plant name AND sulphotransferase', 'Plant name AND methylation', 'Plant name AND methyltransferase', 'Plant name AND acetylation', 'Plant name AND n-acetyltransferase', 'Plant name AND P-glycoprotein' or 'Plant name AND P-gp" (Tables 1-3 to 1-11). EndNote web was the citation tool used to manage and organize all the references collected.

It is important to note that RENISUS encompasses native and exotic adapted medicinal plant species and that we are aware of the chemical variability of the materials, which eventually may be harmonised by pharmacopoeial monographs of their respective countries.

Also, same plant species can present different results in the literature. This could be explained by the using plants collected in different regions, places and time of the year for example. Also, observed effects may vary due to the extraction procedure used for the conduction of the experiment, as well as the models selected for the study.

Table 1- 3 Medicinal plants listed in RENISUS with reported effects on CYP1A2

Plant species/Family	Effects on CYP1A2	References
<i>Allium sativum</i> L. (Amaryllidaceae)	+	(Le Bon et al., 2003)
<i>Curcuma longa</i> L. (Zingiberaceae)	+, -	(Thapliyal et al., 2002, Volak et al., 2013)
<i>Eucalyptus globulus</i> Labill. (Myrtaceae)	-	(Unger and Frank, 2004)
<i>Glycine max</i> (L.) Merr. (Leguminosae)	-	(Shon and Nam, 2004)
<i>Harpagophytum procumbens</i> DC. Ex (Pedaliaceae)	NE, -	(Modarai et al., 2011b, Unger and Frank, 2004)
<i>Mentha x piperita</i> L. (Lamiaceae)	-	(Unger and Frank, 2004)
<i>Phyllanthus amarus</i> Schumach. & Thonn. (Phyllanthaceae)	-	(Hari Kumar and Kuttan, 2006)
<i>Punica granatum</i> L. (Lythraceae)	-	(Faria et al., 2007a)
<i>Trifolium pratense</i> L. (Leguminosae)	-	(Unger and Frank, 2004)

—, Inhibition of enzyme expression; + Induction of enzyme expression;
NE, No effect

Table 1- 4 Medicinal plants listed in RENISUS with reported effects on CYP2C9

Plant species/Family	Effects on CYP2C9	References
<i>Allium sativum</i> L. (Amaryllidaceae)	-, +	(Ho et al., 2010, Foster et al., 2001)
<i>Eucalyptus globulus</i> Labill. (Myrtaceae)	-	(Unger and Frank, 2004)
<i>Harpagophytum procumbens</i> DC. Ex (Pedaliaceae)	NE, -	(Modarai et al., 2011b)
<i>Mentha x piperita</i> L. (Lamiaceae)	-	(Unger and Frank, 2004)
<i>Punica granatum</i> L. (Lythraceae)	-	(Hanley et al., 2012)
<i>Trifolium pratense</i> L. (Leguminosae)	-	(Unger and Frank, 2004)
<i>Zingiber officinale</i> Roscoe (Zingiberaceae)	-	(Kimura et al., 2010)

—, Inhibition of enzyme expression; + Induction of enzyme expression;
NE, No effect

Table 1- 5 Medicinal plants listed in RENISUS with reported effects on CYP2C19

Plant species/Family	Effects on CYP2C19	References
<i>Allium sativum</i> L. (Amaryllidaceae)	-	(Foster et al., 2001)
<i>Eucalyptus globulus</i> Labill. (Myrtaceae)	-	(Unger and Frank, 2004)
<i>Harpagophytum procumbens</i> DC. Ex (Pedaliaceae)	NE	(Modarai et al., 2011b)
<i>Mentha x piperita</i> L. (Lamiaceae)	-	(Unger and Frank, 2004)
<i>Trifolium pratense</i> L. (Leguminosae)	-	(Unger and Frank, 2004)

—, Inhibition of enzyme expression;
NE, No effect

Table 1- 6 Medicinal plants listed in RENISUS with reported effects on CYP2D6

Plant species/Family	Effects on CYP2D6	References
<i>Allium sativum</i> L. (Amaryllidaceae)	NE	(Markowitz et al., 2003)
<i>Eucalyptus globulus</i> Labill. (Myrtaceae)	-	(Unger and Frank, 2004)
<i>Harpagophytum procumbens</i> DC. Ex (Pedaliaceae)	NE, -	(Modarai et al., 2011b)
<i>Mentha x piperita</i> L. (Lamiaceae)	-	(Unger and Frank, 2004)
<i>Phyllanthus amarus</i> Schumach. & Thonn. (Phyllanthaceae)	-	(Hari Kumar and Kuttan, 2006)
<i>Punica granatum</i> L. (Lythraceae)	-	(Usia et al., 2006)
<i>Trifolium pratense</i> L. (Leguminosae)	-	(Unger and Frank, 2004)

—, Inhibition of enzyme expression;
NE, No effect

Table 1- 7 Medicinal plants listed in RENISUS with reported effects on CYP2E1

Plant species/Family	Effects on CYP2E1	References
<i>Allium sativum</i> L. (Amaryllidaceae)	-	(Le Bon et al., 2003)
<i>Curcuma longa</i> L. (Zingiberaceae)	NE	(Salama et al., 2013)
<i>Glycine max</i> (L.) Merr. (Leguminosae)	NE	(Shon and Nam, 2004)
<i>Momordica charantia</i> L. (Cucurbitaceae)	-	(Raza et al., 1996)
<i>Phyllanthus amarus</i> Schumach. & Thonn. (Phyllanthaceae)	-	(Hari Kumar and Kuttan, 2006)
<i>Phyllanthus urinaria</i> L. (Phyllanthaceae)	-	(Shen et al., 2008)
<i>Punica granatum</i> L. (Lythraceae)	-	(Faria et al., 2007a)

—, Inhibition of enzyme expression;
NE, No effect

Table 1- 8 Medicinal plants listed in RENISUS with reported effects on CYP3A

Plant species/Family	Effects on CYP3A	References
<i>Allium sativum</i> L. (Amaryllidaceae)	NE, -, *, *, *	(Hajda et al., 2010, Foster et al., 2001)
<i>Chamomilla recutita</i> (L.) <i>Rauchert</i> (Compositae)	ND *, *, -, *	(Budzinski et al., 2000)
<i>Curcuma longa</i> L. (Zingiberaceae)	ND *, *, NE *	(Graber-Maier et al., 2010)
<i>Eucalyptus globulus</i> Labill. (Myrtaceae)	ND *, *, -, *	(Unger and Frank, 2004)
<i>Foeniculum vulgare</i> Mill. (Apiaceae)	ND *, *, -, *	(Subehan et al., 2006, Subehan et al., 2007)
<i>Harpagophytum procumbens</i> DC. Ex (Pedaliaceae)	ND *, *, NE, - *	(Modarai et al., 2011b, Unger and Frank, 2004)
<i>Mentha x piperita</i> L. (Lamiaceae)	ND *, *, -, *	(Unger and Frank, 2004)
<i>Momordica charantia</i> L. (Cucurbitaceae)	ND *, *, -, *	(Raza et al., 1996)
<i>Phyllanthus amarus</i> Schumach. & Thonn. (Phyllanthaceae)	-, *, *, *	(Hari Kumar and Kuttan, 2006)
<i>Punica granatum</i> L. (Lythraceae)	-, *, *, *	(Faria et al., 2007a)
<i>Trifolium pratense</i> L. (Leguminosae)	ND *, *, -, *	(Budzinski et al., 2000)
<i>Uncaria tomentosa</i> (Willd. ex Schult.) DC. (Rubiaceae)	ND *, *, -, *	(Budzinski et al., 2000)
<i>Zingiber officinale</i> Roscoe (Zingiberaceae)	ND *, *, -, *	(Kimura et al., 2010)

—, Inhibition of enzyme expression; **NE**, No effect, **ND**, No data

*, CYP3A4, *, CYP3A5, *, CYP3A7

Table 1- 9 Medicinal plants listed in RENISUS with reported effects on GSH levels

Plant species/Family	Effects on GSH levels	References
<i>Achillea millefolium</i> L. (Compositae)	+	(Potrich et al., 2010)
<i>Allium sativum</i> L. (Amaryllidaceae)	+	(Ip and Lisk, 1997)
<i>Aloe vera</i> (L.) Burm.f./ <i>Aloe barbadensis</i> Mill. (Xanthorrhoeaceae)	-,+	(Hegazy et al., 2012, Kaithwas et al., 2011)
<i>Anacardium occidentale</i> L. (Anacardiaceae)	+	(Singh et al., 2004)
<i>Baccharis trimera</i> (Less.) DC.(Compositae)	-	(Nogueira et al., 2011)
<i>Bauhinia forficata</i> Link.(Leguminosae)	-	(Damasceno et al., 2004)
<i>Bauhinia variegata</i> L.(Leguminosae)	+	(Raj Kapoor et al., 2006)
<i>Calendula officinalis</i> L. (Compositae)	+	(Preethi and Kuttan, 2009)
<i>Chamomilla recutita</i> (L.) Rauchert (Compositae)	+	(Al-Hashem, 2010)
<i>Croton cajucara</i> Benth.(Euphorbiaceae)	+	(Rabelo et al., 2010)
<i>Curcuma longa</i> L. (Zingiberaceae)	+	(Rong et al., 2012)
<i>Cynara scolymus</i> L.(Compositae)	+,NE	(Miccadei et al., 2008)
<i>Foeniculum vulgare</i> Mill. (Apiaceae)	+	(Zhang et al., 2012b)
<i>Glycine max</i> (L.) Merr. (Leguminosae)	+	(Barbosa et al., 2011)
<i>Mentha pulegium</i> L. (Lamiaceae)	+	(Alpsoy et al., 2011)
<i>Mentha x piperita</i> L.(Lamiaceae)	+	(Sharma et al., 2007)
<i>Mikania glomerata</i> Spreng (Asteraceae)	NE	(Barbosa et al., 2012)
<i>Momordica charantia</i> L. (Cucurbitaceae)	+	(Raza et al., 1996, Raza et al., 2000)
<i>Phyllanthus amarus</i> Schumach. & Thonn. (Phyllanthaceae)	+	(Karuna et al., 2009, Kumar and Kuttan, 2004, Kumar and Kuttan, 2005, Maity et al., 2013)
<i>Phyllanthus niruri</i> L. (Phyllanthaceae)	+	(Manjrekar et al., 2008, Bhattacharjee and Sil, 2006)

Plant species/Family	Effects on GSH levels	References
<i>Psidium guajava</i> L. (Myrtaceae)	+	(Tandon et al., 2012)
<i>Ruta graveolens</i> L. (Rutaceae)	+	(Ratheesh et al., 2011)
<i>Zingiber officinale</i> Roscoe (Zingiberaceae)	+,NE	(Ajith et al., 2007)

+, Increase GSH levels; —, Decrease GSH levels; **NE**, No effect;

Table 1- 10 Medicinal plants listed in RENISUS with reported effects on UGT

Plant species/Family	Effects on UGT	References
<i>Allium sativum</i> L. (Amaryllidaceae)	+	(Ip and Lisk, 1997)
<i>Curcuma longa</i> L. (Zingiberaceae)	-	(Naganuma et al., 2006)

+, Increased enzyme activity; —, Decreased enzyme activity

Table 1- 11 Medicinal plants listed in RENISUS with reported effects on P-gp efflux activity

Plant species/Family	Effects on P-gp efflux activity	References
<i>Achillea millefolium</i> L. (Compositae)	-	(Haidara et al., 2006)
<i>Allium sativum</i> L. (Amaryllidaceae)	+	(Hajda et al., 2010)
<i>Curcuma longa</i> L. (Zingiberaceae)	NE, -	(Graber-Maier et al., 2010, Ampasavate et al., 2010)

+, Increased efflux activity; —, Decreased efflux activity; **NE**, No effect

1.10 AIM OF THE THESIS

As highlighted earlier the SUS aims to include more herbal medicines to RENAME within the coming years. However, the intake of those herbal medicines by the patients of SUS could potentially cause HDI if they are coadministered with essential medicines. Due to the types of effects that herbal medicines could cause in phase I and phase II metabolising enzymes and P-gp, the present study aims to answer whether selected RENISUS herbal medicines are able to modulate designated metabolic and transporter targets *in vitro* in order to forestall HDI with some essential medicines available in SUS.

To achieve this, we carried out an experimental evaluation of the *in vitro* effects of twenty-four plant extracts from twenty-three RENISUS medicinal plant species selected based on data collected in our literature analysis on different cell detoxification and transport mechanisms; as a means of identifying potential HDI. Parts of this experimental evaluation have been previously published as an original research article (Mazzari et al., 2016).

2 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Cell Culture. HepG2 and Caco-2 cell lines were from Sigma Aldrich at passages 100 and 43 respectively. HeLa cells were donated by Dr. Paul Webb from the Houston Methodist Institute for Technology at passage 20. All cell culture reagents were from Gibco® Invitrogen unless otherwise stated. Vincristine 2mg/mL was purchased from Hospira Ltd.

HPTLC analysis. Water, dichloromethane (>99.8%, contains amylene as a stabilizer), ethyl acetate (>99.7%) and methanol (>99.9%) all ChromasolvPlus for HPLC provided by Sigma Aldrich. Acetic acid (glacial) analytical reagent grade provided by Fisher Scientific. Formic Acid 98% provided by Rectapur® VWR. Caffeic acid was from Kotech-light Laboratories LTD. Rutin hydrate, quercetin dehydrate and gallic acid all from Sigma Aldrich. Diphenylborinic acid 2-aminoethyl ester (98%) and coumarin laser grade (99% UV-Vis) were provided by ACROS organics. Luteolin (HPLC grade) was from Extrasynthèse. Chlorogenic acid provided by Cayman Chemical Co. Polyethylene glycol 4000 grade was from Fisher Scientific.

HPLC analysis. All solvents (water HPLC gradient grade, glacial acetic acid and methanol) were purchased from Fisher Scientific UK. Caffeic acid, catechin, rutin, quercetin, and chlorogenic acid were from Sigma Aldrich.

Cell viability assays. Sulphorhodamine B (SRB), neutral red (NR), (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT), trichloroacetic acid (TCA), glacial acetic acid, 96% ethanol, hydrochloric acid (HCl), Isopropanol and Tris base were from Sigma Aldrich.

CYP3A4 mRNA gene expression assay. Oligonucleotide primers were custom-synthesized by Invitrogen Life Technologies and Sigma Aldrich. TRIzol® (Total RNA Isolation Reagent), Oligo (dT) 12-18 primers, M-MLV Reverse Transcriptase, RNaseOUT, DNase I Amplification Grade and 100mM dNTP set were purchased from Invitrogen Life Technologies. SYBR Green (2xqPCR Master Mix premixed with SYBRgreen) was obtained from Amethyst reagents (Cambridge Bioscience). Rifampicin (RIF) and DMSO were purchased from Sigma Aldrich.

hPXR assay. Lipofectamine® 2000 was purchased from Invitrogen. RIF and Luciferase assay system were obtained from Sigma Aldrich and Promega respectively.

Intracellular Glutathione assay. Buthionine sulfoximine (BSO), L-glutathione reduced, glutathione reductase (GR), 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (β -NADPH), Triton-X and sulfosalicylic acid (SSA) were from Sigma Aldrich.

GGT activity assay. γ -glutamyl-*p*-nitroanalide (GpNA), *p*-nitroaniline, glycyl-glycine (Gly-Gly), Tris base and acivicin were from Sigma Aldrich.

Rh-123 uptake assay. Rh-123 was from Sigma Aldrich. Verapamil (Securon IV 2.5mg/mL) was from Abbott Laboratories Ltd.

2.2 PREPARATION OF PLANT MATERIALS

2.2.1 Selection criteria and ethical clearance

The medicinal plants used for our *in vitro* preclinical studies were selected according to the results found in the literature review (chapter 1). Originally, we planned to work with all plant species where no preclinical published data had been found. However, it was not possible to obtain all the plant species from the main natural products suppliers in Brazil. Therefore we conducted our work with 23 plant species, which were readily available for purchasing and collection with three different suppliers in Brazil: Santosflora Comércio de Ervas (Mairiporã, São Paulo, Brazil), Florien (Piracicaba, São Paulo, Brazil) and “Farmácia Viva Brasília” (Brasília, DF – Brazil), via UnB.

In order to conduct our research, we had to first to deal with a series of regulations to access the Brazilian genetic heritage ‘component’, which is controlled by the *Conselho de Gestão do Patrimônio Genético* (Genetic Heritage Council) or CGEN. According to CGEN, in order to purchase and/or collect medicinal plants in Brazil for research purposes and to transport them to the UK, we needed to first to establish an institutional agreement between UCL and a Brazilian institution that would be legally responsible for the whole process (Appendix 7.1). We contacted several Brazilian universities exploring

a possible collaboration. We eventually received a positive feedback from Dr. Damaris Silveira from the Faculty of Life Sciences of the University of Brasília (UnB) (Brasília – Distrito Federal) who was willing to help us with all the bureaucratic processes.

The second step was to elaborate the project and send a clear plan of it to CGEN and to the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (Brazilian Institute of Environment and Renewable Natural Resources) or IBAMA. The plan had details of the type of research to be carried out with the selected plant extracts and included materials, methodologies and a timetable for the execution of the entire project (Appendix 7.2).

The third (and last) step was the production of a material transfer agreement (MTA) document, which was countersigned by the legal representatives of University College London (UCL) and UnB (Appendix 7.3). All documents were prepared by us and sent to Brazil between November 2013 and March 2014.

2.2.2 Processing of plant materials

The plants were grown and processed according to good manufacturing practices established by ANVISA, ensuring validity and quality (Carvalho et al., 2014). All whole plant handling and extraction were performed at UnB. All used parts of the plants were subjected to a 20-minute infusion in order to mimic traditional use (Academia Nacional de Medicina (Madrid Spain), 1954). For infusion, boiled distilled water (300mL) was added to the plant material (20g) with continuous stirring for 15 minutes on a laboratory hot plate magnetic stirrer (IKA WERKE labortechnik electric stirrer). All extracted plant samples were allowed to stand until cold before filtering with a Buchner flask. All the filtered extracts were frozen in round bottom flasks and then freeze dried (Edwards Pirani 501 Savant super modulo freeze drier) to obtain the dried extract. The lyophilised extracts were immediately sent to UCL School of Pharmacy and stored at -18°C throughout the studies.

2.3 PHYTOCHEMICAL ANALYSIS

2.3.1 HPTLC analysis

Extracts were diluted to a concentration of 50mg/mL in methanol. Control compounds were made at a concentration of 1mg/mL, also diluted in methanol. A CAMAG Linomat 5 was used to apply 5 μ L of the samples to TLC silica gel 60 F254 aluminium sheets. The plates were developed using a CAMAG ADC2 automatic developing chamber. The method included 30-second pre-drying, 10 minutes humidity control using magnesium chloride to 48.3% relative humidity and 20 minutes saturation time, using saturation pads all done at 25.2°C. The mobile phase used was ethyl acetate: formic acid: water (82:9:9). During development, the solvent front was allowed to migrate 80mm before a drying time of 5 minutes. For derivatization, we used natural products reagent (NPR) followed by PEG 4000 (Reich and Schibli, 2007). All visualization and analysis were done using CAMAG TLC visualizer both before and after derivatization.

2.3.2 HPLC analysis

Phytochemical analysis of all samples of the freeze-dried plant extracts was carried out using a high-performance liquid chromatography (HPLC) system- Agilent 1200 series equipped with an ultraviolet/visible (UV-Vis) diode array detector to obtain a 'fingerprint' of the samples. The conditions used for the analysis have previously been used for the detection of phenolic compounds in plant samples (Giner et al., 1993).

The stationary phase was a Phenomenex® synergi Polar-RP80A column (250mm x 4.6mm, I.D = 4 μ m) maintained at 40°C and protected with a Nova-Pak® C18 guard column. The mobile phase was a binary system consisting of (A) water-glacial acetic acid (99.8:0.2, v/v) and (B) methanol-glacial acetic acid (99.8:0.2, v/v). The gradient elution program for the mobile phase was 10-20% B (0-5 minutes), 20-50% B (5-65 minutes), 50-80% B (65-75 minutes) and 80-100% B (75-80 minutes) at a flow rate of 1mL/minute. The injected volume of the sample was 10 μ L and the UV-Vis spectra were recorded

in the range 190 to 400nm, while chromatographic peaks were monitored at 254 and 360nm.

The plant extracts were prepared as 25mg/mL solutions in either micro-filtered double distilled water or HPLC-grade methanol. Five plant phenolic compounds: caffeic acid, catechin, rutin, quercetin, and chlorogenic acid were used as standards at concentrations between 0.5 and 1mg/mL in methanol, depending on the intensity of the peak. Samples were filtered through a 0.45 μ m syringe filter (Millipore Millex® PTFE) before the analysis.

2.4 IN VITRO STUDIES

2.4.1 Mammalian cell culture techniques

2.4.1.1 Human liver hepatocellular carcinoma (HepG2), colorectal carcinoma cells (Caco-2) and cervix adenocarcinoma (HeLa) cells

Human liver hepatocellular carcinoma, or simply HepG2, is an accepted cell line model commonly used for *in vitro* drug metabolism studies as they keep some specialised functions when compared to human hepatocytes (HH). HepG2 also preserves the activity of several phase I, phase II, and antioxidant enzymes (Vidyashankar et al., 2010). Besides, HepG2 cells have the advantage of ready availability and assurance in reproducibility of experiments (Hewitt and Hewitt, 2004). HepG2 cells were used in our study to evaluate the CYP3A4 mRNA gene expression, intracellular GSH levels, and GGT activity.

Caco-2 cells are widely used for *in vitro* drug transport studies. The capacity of Caco-2 to form monolayers with morphological and functional similarities to the human intestinal epithelium, meant the cell line received recognition from the Food and Drug Administration (FDA) as a viable model that replicates human intestinal absorption (Chen et al., 2016). Our drug transport study is focused on the P-gp efflux activity, and we therefore used vincristine resistant Caco-2 cells (Caco-2 VCR) model, which displays a more pronounced efflux activity mechanism than normal Caco-2 (Eneroth et al., 2001).

Henrietta Lacks (HeLa) cervix adenocarcinoma cells were discovered in 1951 and became the first human cancer cell line immortalised in tissue culture

(Lucey et al., 2009). HeLa is one of the most versatile cancer cell lines and it is widely used in molecular biology, especially due to its easy and efficient transfection with plasmids using Lipofectamine (Nikcevic et al., 2003). Among some uses in molecular biology, transfected HeLa cells have been the choice of researchers for evaluating hPXR-dependent gene expression, including proteins like CYPs (Lemaire et al., 2006). HeLa cells were therefore our choice for studying the effect of four RENISUS plant extracts on hPXR.

2.4.1.2 Resuscitation of frozen cell stocks

Cell lines are usually stored in frozen form (in liquid nitrogen) when not in use. The cells used in this study were in this form when purchased. To resuscitate them, the cells were thawed rapidly at 37°C by placing the vial of cells in a pre-heated water bath. The thawed cells were then diluted slowly into a centrifuge tube containing the appropriate warm growth media to eliminate DMSO, which is toxic to cells. DMSO is used as a cryoprotectant to prevent crystallisation, which will otherwise cause cells to lyse during cryopreservation. The tubes were then centrifuged (Biofuge primo R Heraeus centrifuge) for 5 minutes at 1000rpm to separate the cell pellet from the culture media. Inside the laminar flow cabinet, the media was removed from the centrifuge tube and the cells were properly resuspended in 15mL of pre-warmed complete media. They were then transferred to a culture flask pre-labelled with the name of the cell line, passage number, and date. An inverted microscope (Nikon TMS 10/0.25 lens) was used to check the morphology of the cells before placing in an incubator (Ezuruike, 2015).

2.4.1.3 Subculture/maintenance

HepG2, Caco-2, and HeLa cells were stocked in DMSO and frozen in liquid nitrogen until they were used. When the cells were needed they were taken out of the liquid nitrogen and defrosted in the water bath (37°C) for approximately 4 minutes and then transferred to a flask containing 15mL of pre-warmed media and centrifuged at 1000rpm for 5 minutes at 18°C. The media (supernatant) was removed and the cells were re-suspended with 5mL of the same media and then transferred to a cell culture flask containing 15mL of pre-

warmed media and incubated. The cells were checked regularly in order to observe their growing. Cells were incubated in culture flasks (TPP, surface area 75cm²) containing 20mL of HepG2 medium (MEM Alpha + GlutaMax 1x) or Caco-2 and HeLa medium (DMEM Alpha + Glutamax 1x), 1% (v/v) penicillin-streptomycin antibiotic (10,000 units/mL penicillin and 10,000 µg/mL streptomycin) and 10% heat-inactivated foetal bovine serum-EU. Caco-2 cells were also incubated with vincristine (20nM). The media was kept in the fridge (2°C - 8°C) and prior to its use in cells, the media was placed for 30 minutes in the water bath (37°C) (Ezuruike, 2015).

2.4.1.4 Cell passage (propagation)

When the cells reached around 70% of confluence the cell passing procedure was performed. The old media was aspirated from the cell culture flask and the cells were washed with 5mL of phosphate saline buffer (PBS) to remove the remaining media. After removing the PBS, 1.2mL of 0.25% (w/v) trypsin/EDTA was added to the flask, it was then placed in the incubator until the cells detached. Afterwards, the volume of media was added according to the split ratio desired and the new flasks were then placed in the incubator again allowing the cells to grow (Ezuruike, 2015).

2.4.1.5 Cell counting

The confluence of the flask was checked and then the media was aspirated. The cells were then rinsed with 5mL of PBS and 1.2mL of trypsin/EDTA was added to detach the cells. The flask was put in the incubator for approximately 3 minutes. Afterwards, the flask was placed in the laminar flow cabinet and 190µL of trypan blue stain 0.4% was placed in an eppendorf plus 10µL of the cell suspension. The microcentrifuge tube was mixed on a rotamixer for a few seconds and 10µL of the trypan blue cell suspension were added to each side of a Neubauer haemocytometer that was placed in an inverted microscope (Nikon TMS). The cells were then counted and plated out according to the number of cells desired per well (Ezuruike, 2015).

2.4.2 Cell Viability Assays

2.4.2.1 The SRB assay

2.4.2.1.1 Background

Toxicity of HepG2 cells was assessed using SRB assay. The SRB (Figure 2-1) assay is commonly applied for testing cytotoxicity in a wide range of cell lines. The assay relies on the binding of the negatively charged pink aminoxanthine dye by protein components of cells that have been treated and fixed with TCA. The sulphonic groups present in the SRB dye has the ability to bind to basic amino acids residues under mildly acidic conditions. The dye is able then to dissociate under basic conditions. The number of viable cells is detected according to the amount of dyed ones, which is proportional to the cell mass due to the stoichiometric binding of the SRB dye to fixed cells. When the cells are lysed the colour becomes more intense and the absorbance increases (Houghton et al., 2007). The reason why this assay was selected for cytotoxicity analysis is that SRB is a quite reliable method and the results correlate well with those of tetrazolium dye MTT for example (Vichai and Kirtikara, 2006).

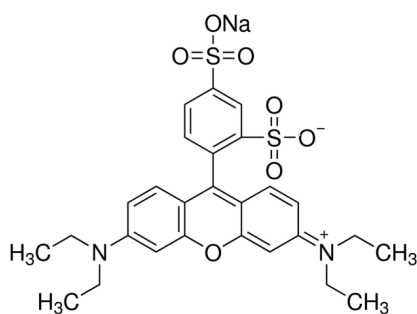


Figure 2- 1 SRB chemical structure.

2.4.2.1.2 Experiment protocol

The method used for this assay is as described in Houghton and coworkers (2007) with slight modifications. After 24 hours incubation of HepG2 cells (2×10^4 cells/well) with the plant extracts (100 μ g/mL) and serial dilutions of the positive control ethanol, media containing the samples was removed and cells were fixed with 100 μ L of cold 40% w/v TCA solution in deionized water.

The plates were incubated at 4°C for 1 hour and then immersed five times in tap water. The TCA-fixed cells were stained by adding 100µL of SRB solution (0.4 % SRB in 0.1% glacial acetic acid) and left at room temperature for 1 hour. Afterwards, the plates were quickly rinsed four times with 1% acetic acid and flicked to remove unbound dye and then left to air-dry overnight. After drying completely, the protein bound SRB was solubilised by adding 100µL of Tris base buffer solution (10mM) to each well. The plates were agitated in an orbital shaker for 30 minutes (IKA MS3 basic shaker). The optical density was measured at 492nm by using a microplate plate reader Infinite M200 (Tecan Trading AG, Switzerland) and the viability was calculated as follows: % viability = $(O.D_S - O.D_B / O.D_M - O.D_B) \times 100$; where $O.D_S$ is the optical density of the sample (either drug or plant extract), $O.D_B$ is the optical density of the blank wells without cells, $O.D_M$ is the optical density of the wells of the positive control containing only complete media (Houghton et al., 2007).

2.4.2.2 Neutral Red Uptake assay

2.4.2.2.1 Background

Toxicity of Caco-2 VCR cells was determined using the neutral red assay. The assay was based on the uptake of neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) (Figure 2-2), a supravital dye, and its accumulation in the lysosomes of viable uninjured cells. The uptake of the dye depends on the cell's capacity to maintain pH gradients, through the production of ATP. The dye is weakly cationic and at physiological pH, presents a net charge close to zero, enabling it to penetrate the cell membranes by non-ionic passive diffusion and concentrates in the lysosomes. Inside the lysosome, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus the dye becomes charged and is retained, where it binds to anionic and/or phosphate groups of the lysosomal matrix (Repetto et al., 2008).

Extraction of the dye from the viable cells was done using an acidified ethanol solution, and the absorbance of the solubilised dye is quantified spectrophotometrically at 540nm. Quantification of the dye extracted from the cells have been shown to be linear with cell numbers, both by direct cell counts

and by protein determinations of cell populations (Weyermann et al., 2005). Given that the toxicity of the extracts is inversely proportional to the viability of the cells, more toxic extracts result in fewer cells being available for neutral red uptake. The neutral red assay has been identified as a reliable *in vitro* assay that can be used to estimate *in vivo* starting doses for acute toxicity based on its low coefficient of variation from several inter- and intra-laboratory experiments (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), 2001).

Originally, SRB was the cytotoxicity method chosen for Caco-2 VCR cells. However, the washing steps were washing away the cell monolayer and therefore we had to choose a less aggressive method to avoid chances of cell detachment from the 96-well plate, hence the selection for this study.

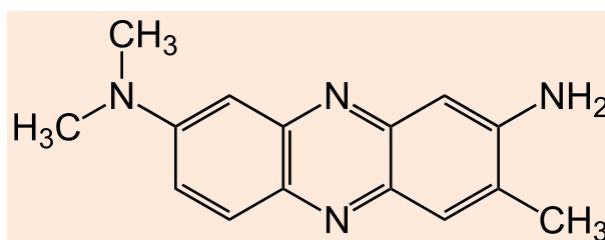


Figure 2- 2 Chemical structure of neutral red dye.

2.4.2.2.2 Experiment protocol

The method used for this assay is as described in Repetto and coworkers (2008) with slight modifications. Caco-2 VCR cells (1×10^4 cells/well) were plated out in each of the inner wells of a sterile, flat-bottom 96-well plate. The plate was then incubated overnight at 37°C, 5% CO₂/95% O₂.

The next day, the plant extracts to be tested (100µg/mL), including the serial dilutions of the positive control hydrogen peroxide were made in complete media from the appropriate stock solutions.

After 24 hours incubation, the growth media was carefully aspirated from each well of the microplate and replaced with 200µL of the test solution in replicates. Complete media without drug was added to some of the wells with cells as well as the outer wells without cells, in order to act as a positive control and a blank respectively. The microplate was then incubated for 24 hours.

Neutral red (40 μ g/mL) in complete media was prepared in a centrifuge tube from a stock solution of 4mg/mL in PBS and incubated overnight, wrapped in foil paper at the same temperature as the cells.

Subsequently, the neutral red solution was centrifuged for 10 minutes at \approx 1800 rpm to remove precipitated dye crystals. The microplate was then removed from the incubator and the media in each of the wells was gently aspirated off and replaced with 100 μ L of the neutral red solution in each well of the plate and then incubated for another 2 hours. After incubation, each well of the plate was gently rinsed with PBS (150 μ L) and the washing solution removed by decanting or gently tapping the plate (Ezuruike, 2015).

150 μ L of neutral red destain solution (96% ethanol, deionised water, glacial acetic acid; 50%:49%:1%) was added to each well of the plate after the washing step. The plate was immediately shaken on a microplate shaker for at least 10 minutes until the neutral red had been completely extracted from the cells and formed a homogenous solution. The optical density (O.D)/absorbance of the neutral red extract was read out in the plate reader at 540nm. The percentage viability for each of the concentrations of the drug/extract was estimated as previously stated in section 2.4.2.1.2 (Repetto et al., 2008).

2.4.2.3 The MTT assay

2.4.2.3.1 Background

Toxicity of HeLa cells was measured using MTT assay. When the yellow water soluble tetrazolium salt reaches the mitochondria, the succinate dehydrogenase enzyme cleaves the tetrazolium ring, converting the salt into insoluble purple formazan crystals. As the reaction is mitochondria-dependant, the formazan crystals will only be formed in viable cells and will remain there due to the impermeability of the compound to the cell membranes. Only after the addition of an acidified isopropanol solution the product is liberated and can readily be detected and quantified by a colorimetric method (Fotakis and Timbrell, 2006). MTT was chosen for cytotoxicity analysis of the HeLa cells because it is one of the most standard and popular methods for such

assessment in cells that are submitted to transfection (Bednarek et al., 2008, Li et al., 2006, Cheng et al., 2007).

2.4.2.3.2 Experiment protocol

The method used for this assay is as described in José Ruiz and coworkers (2006) with some modifications. After 24 hours incubation of HeLa cells (2×10^4 cells/well) with the plant extracts (100 μ g/mL) and serial dilutions of the positive control ethanol, media containing the samples was removed and 200 μ L of fresh media containing 50 μ L of MTT (1mg/mL) were added to each well. After 4 hours incubation, the solution was carefully aspirated and replaced by 150 μ L of MTT solubilisation solution (0.04M HCl in isopropanol). Plates were shaken until crystals were completely dissolved and absorbance was measured at 570nm in the plate reader and the viability for each of the concentrations of the drug/extract was estimated as previously described in section 2.4.2.1.2 (José Ruiz et al., 2006).

2.4.3 CYP3A4 mRNA gene expression assay

2.4.3.1 Background

Changes in mRNA expression in a cell caused by a xenobiotic can be measured several ways, but quantitative PCR (qPCR) is the “gold standard” technology to quantify nucleic acids. qPCR is a very sensitive and robust technique that collects fluorescent signals from one or more polymerase chain reactions over a range of cycles (Dorak, 2007).

Briefly; after the mRNA is extracted from a tissue or cell, it needs to be retro-transcribed into complementary DNA (cDNA) and this is accomplished by using a DNase, reverse transcriptase enzyme, an oligoprimers, dNTPs (dATP, gGTP, dCTP, and dTTP), RT buffer and RNase inhibitor. Before the beginning of the cDNA synthesis, mRNA is treated with a DNase in order to digest single – and double (dsDNA) – stranded DNA, by the cleavage of each strand of dsDNA independently. In the presence of Mg^{2+} , DNase cleaves each strand of dsDNA independently and randomly. In the presence of Mn^{2+} , the enzyme cleaves both DNA strands at approximately the same site (Figure 2-3).

The first strand cDNA synthesis occurs by the incubation of the mRNA with a poly(T) tail oligoprimers, which will anneal the poly(A) tail of mRNA in a designed temperature. The reverse transcriptase, RNase inhibitor, dNTPs and RT buffer are added to the reaction and transcription will occur at a certain temperature, which will be assigned according to the reverse transcriptase enzyme used. When the enzyme is deactivated by an increased temperature, the cDNA is formed (Figure 2-4).

The synthesised cDNA is then mixed with a DNA polymerase, a target primer sequence (forward and reverse primers) and a fluorescent dye. The DNA polymerase is an enzyme that helps catalyse the polymerization of the deoxynucleotides into a DNA strand. One of the target primers will anneal to the start of the target DNA and another one will anneal to the end of the sequence. The fluorescent dye will bind to the DNA and the light will be captured and recorded by the system. The more copies of the target gene are present in the sample over a range of cycles (denaturation, annealing, and extension), the higher will be the intensity of the fluorescence signal (Figure 2-5).

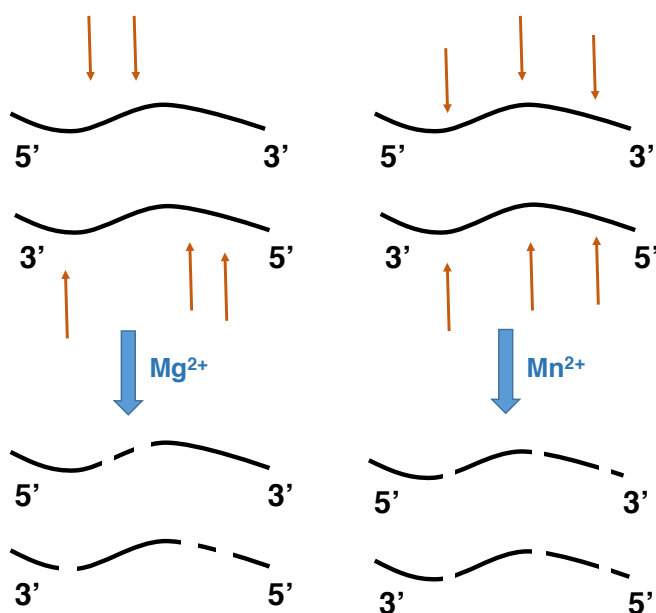


Figure 2- 3 Cleavage of dsDNA by DNase in presence of Mg^{2+} and Mn^{2+} .

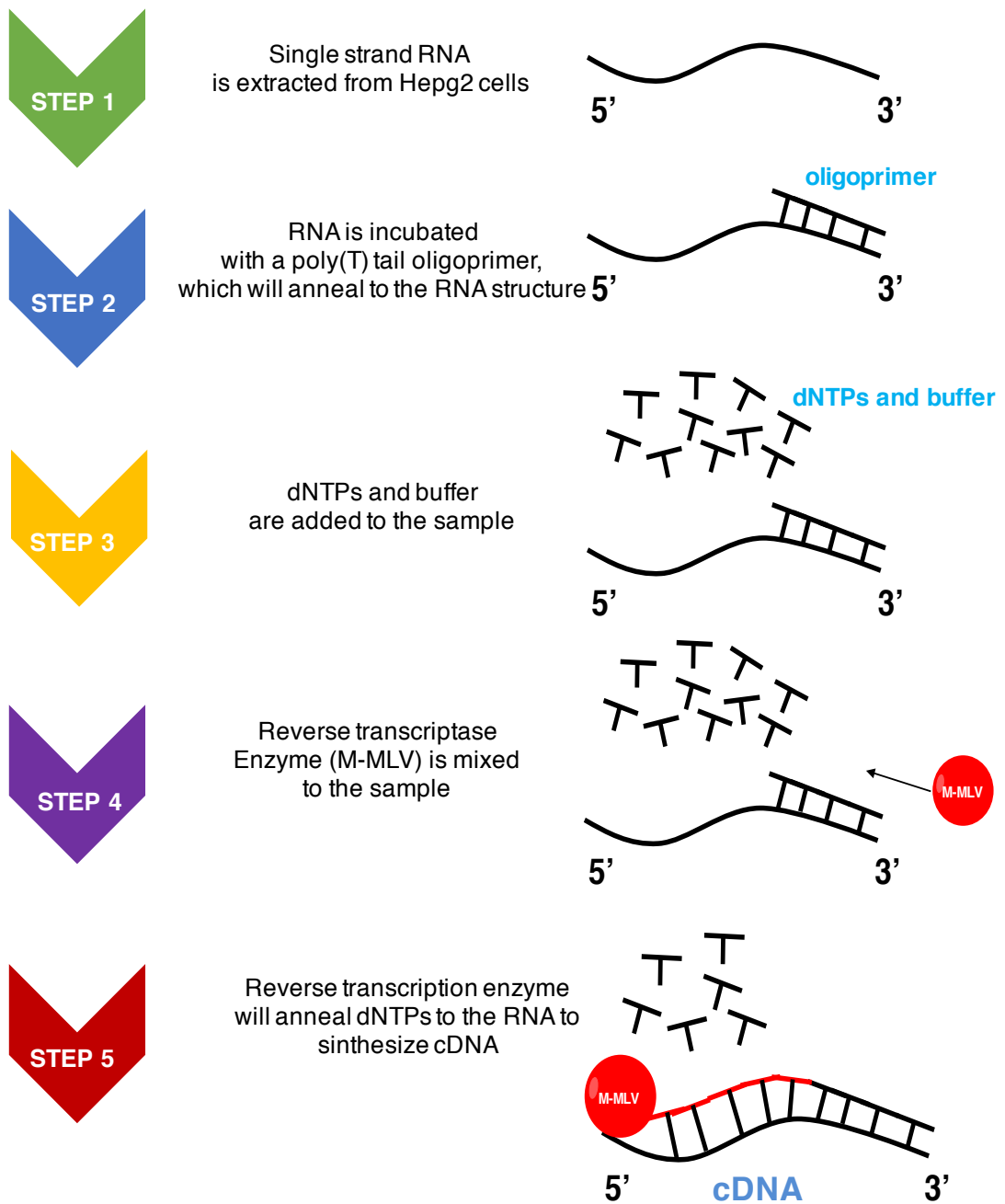


Figure 2- 4 Graphical representation of cDNA synthesis from RNA template. After extraction, RNA is mixed with a oligoprimers, dNTP's and reverse transcriptase enzyme (M-MLV). The oligoprimers is a poly(T) tail sequence, which will anneal to the poly(A) tail of the RNA molecule. When dNTPs, buffer and a reverse transcriptase enzyme (such as M-MLV) is added to the sample, a complementary strand of DNA (or cDNA) is synthesized.

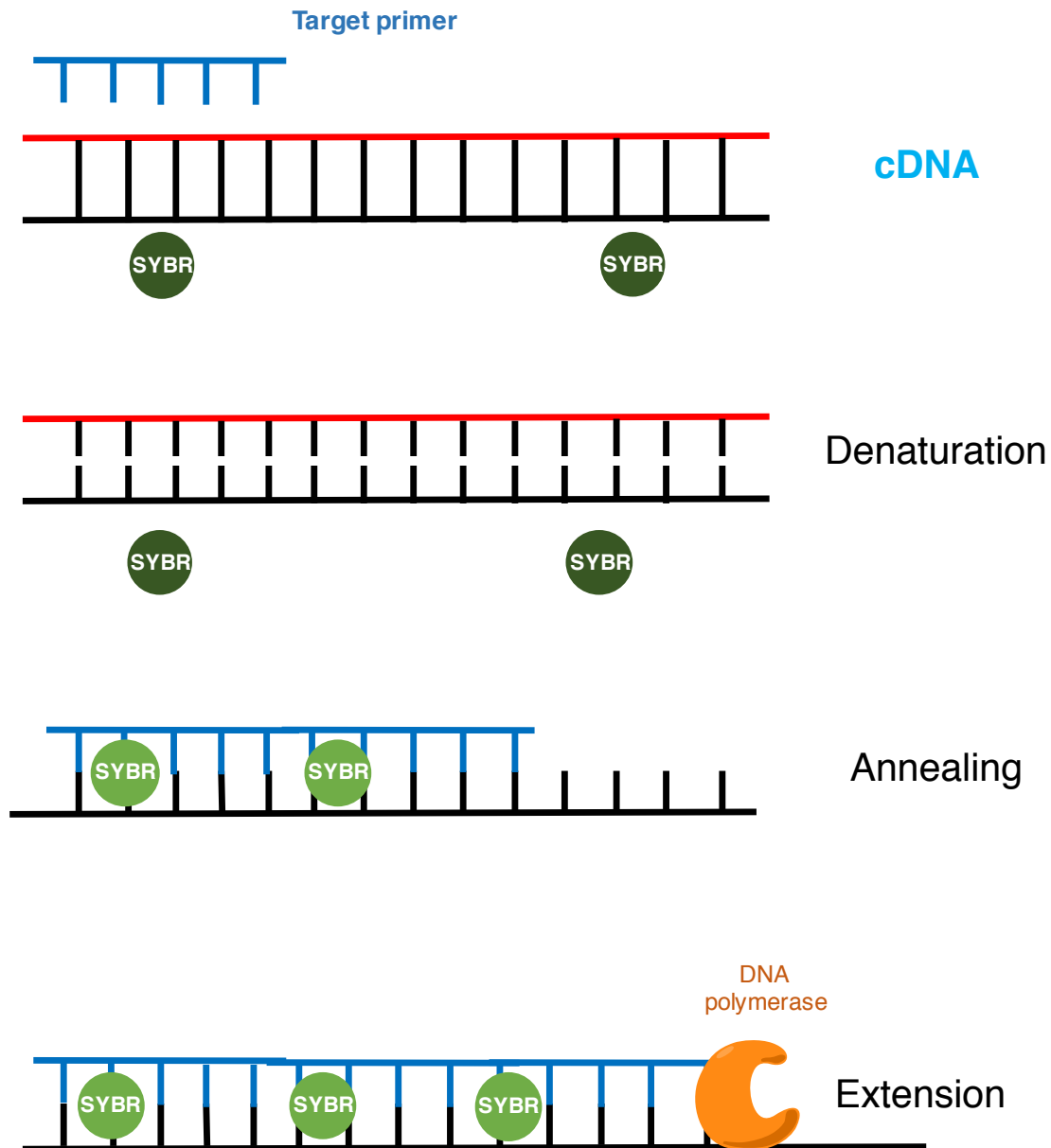


Figure 2- 5 Graphical representation of the addition of a target primer sequence and a Master Mix containing SYBR® Green dye and Taq polymerase enzyme. This reaction results in an increase in fluorescence signal during the PCR analysis. SYBR® Green as a free dye displays very low fluorescence and it will not bind to the single stranded or denaturated DNA. At the annealing stage, when a double-stranded structure is formed, SYBR® Green will bind to it and, as a result, an increase in fluorescent signal is observed. During the extension stage, which is conducted by the enzyme Taq polymerase, the fluorescent signal increases according to the number of SYBR® Greendye molecules bound per double-stranded molecule. This process is repeated in each cycle until it reaches a total of 40 cycles.

2.4.3.2 Experiment protocol

2.4.3.2.1 Total RNA extraction from HepG2 cells

After exposing HepG2 cells (5×10^5 cells/well) to plant extracts ($100\mu\text{g/mL}$) or the CYP3A4 inducer RIF ($50\mu\text{M}$) or the CYP 3A4 inhibitor DMSO 1% for 96 hours, total RNA extraction started by lysing the cells with TRIzol® Reagent (1mL). The homogenised sample was transferred to 1.5mL RNase/DNase free microfuge tubes and incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Chloroform ($200\mu\text{L}$) was added to each sample and the microfuge tubes were centrifuged at $12,000 \times g$ for 15 minutes at 4°C . The mixture separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase, where the RNA remains. The aqueous phase of the samples was transferred to new 1.5mL RNase/DNase free microfuge tubes and 100% isopropanol ($500\mu\text{L}$) was added in order to precipitate the RNA. The tubes were let at room temperature for 10 minutes and centrifuged at $12,000 \times g$ for 10 minutes at 4°C . The supernatant was removed and the RNA pellet was left in the tubes for washing. The washing step was conducted by adding 1mL of 75% ethanol to each tube and the centrifuged at $12,000 \times g$ for 10 minutes at 4°C . This step was repeated twice and after the removal of the ethanol solution, the RNA pellet was air dried for 30 minutes. The RNA was resuspended with RNase free water ($100\mu\text{L}$) and the quantity, quality and integrity of the samples were assessed by agarose gel electrophoresis and NanoDrop, respectively.

2.4.3.2.2 Agarose gel electrophoresis-assessment of the integrity of RNA

The integrity of total RNA from HepG2 cells was assessed by visual inspection of the two rRNAs 28s and 18s on agarose gels. Ethidium bromide ($1\mu\text{L}$) was added to 1% agarose gel solution and the cooled molten agarose solution was poured into the gel tray containing a 10-well foaming comb. After 30 minutes, the gel was set and the tank was filled with 1 x TAE buffer. Gel loading dye ($1\mu\text{L}$) was added to $5\mu\text{L}$ total RNA and this was loaded into each lane.

2.4.3.2.3 NanoDrop reading-assessment of the purity and quality of RNA

The quantity and quality of RNA were determined by adding 1.5 μ L of the RNA sample to the Nanodrop 2000 (Thermo Scientific). Differential readings were made at 230, 260, and 280nm.

2.4.3.2.4 cDNA synthesis

Prior to the reverse transcription, RNA samples were treated with DNase I (1U/ μ L) to avoid genomic contamination. cDNA synthesis started by adding 1 μ g of total RNA to 0.5mL RNase/DNase free microfuge tubes containing 10mM (1 μ L) dNTP Mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH, Oligo (dT)₁₂₋₁₈ (500 μ g/mL) (1 μ L), and sterile distilled water up to 12 μ L volume. The samples were heated at 65°C for 5 minutes and chilled at 4°C for 2 minutes to let the primer anneal to the RNA in an MJ Mini Personal Thermal Cycler (Bio-Rad). 5X First-Strand Buffer (4 μ L), 0.1 DDT (2 μ L) and RNase OUT™ Recombinant Ribonuclease Inhibitor (40 units/ μ L) (1 μ L) were added to the samples tubes and the mixture was incubated at 37°C for 2 minutes. The Moloney murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) (200U/ μ L) (1 μ L) was added to each sample and the tubes were incubated for 50 minutes at 37°C. The reaction was then inactivated by heating the samples at 70°C for 15 minutes.

2.4.3.2.5 RT-qPCR conditions and analysis

CYP3A4 sense strand primer sequence was 5'-CAAGGACAACATAGATCGTTACATATACACACCCTTTGGAAG-3' and the antisense strand primer was 5'-AGCTCAATGCATGTACAGAATCCCCGGTTA-3'. The β -actin gene was used to control for variations in RNA loading within the experimental conditions. The sense strand primer sequence was 5'-CGTACCACTGGCATCGTGAT-3' and the antisense strand primer was 5'-GTGTTGGCGTACAGGTCTTTG-3'. The RT-qPCR was carried out in 96-well plates using a Pikoreal™ Real-Time PCR detection system (Thermo Scientific). Each well contained a final reaction

volume of 10 μ L (5.0 μ L MasterMix with SYBR Green, 2.0 μ L cDNA template diluted appropriately, 0.5 μ L of each primer at a final concentration 0.3mM and 2.0 μ L of RNase/DNase free distilled water). The PCR reaction was performed under the following schema: initial denaturation at 95°C for 2 minutes, then 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C (β -actin) or 60°C (CYP3A4) for 30 seconds, and extension at 72°C for 30 seconds.

At the end of the run, a melting curve was generated by heating the amplicon from 60 to 95°C in order to confirm the specificity of the amplification of each primer pair. All samples were run in quadruplicates. Standard curves were produced to check the PCR efficiency using a five-fold dilution series of cDNA. Efficiency (E) of primer pairs was obtained from the slope of the calibration curve generated. The relative expression was calculated on the basis of 'delta delta Ct' ($\Delta\Delta$ Ct) values. Normalisation of the target gene was achieved by using β -actin as a reference gene.

2.4.4 hPXR activation assay

2.4.4.1 Background

Regulation of CYP3A4 mRNA gene expression is mostly related to the binding of xenobiotics to nuclear receptors, such as the hPXR, which are DNA binding and ligand-regulated transcriptional factor. An interesting feature of hPXR is its ligand-binding domain (LBD) (Xie, 2009). hPXR LBD is hydrophobic and it is able to change its shape in order to better accommodate numerous xenobiotics, such as antibiotics, calcium channel blockers, dietary supplements, environmental pollutants and herbal medicines (Chang, 2009, Shukla et al., 2011).

In the case that no ligand binds to hPXR, CYP3A4 transcription is inhibited by orphan nuclear receptors, such as the small heterodimer partner (SHP) and the silencing mediator of retinoid and thyroid hormone receptors (SMRT). These receptors are transcribed by coactivators, such as steroid receptor coactivator-1 (SRC-1), hepatocyte nuclear factor α (HNF α), and peroxisome proliferator-activated receptor γ coactivator-1- α (PGC-1 α) (Figure 2-6A).

Upon xenobiotic binding, hPXR translocates into the nucleus and blocks the transcription of SHP/SMRT. The ligand-PXR then forms a heterodimer complex with the retinoid X receptor α (RXR α), which will then bind to the xenobiotic responsive enhancer molecule/PXRE (XREM/PXRE) region of the CYP3A4 promoter to initiate the expression of the enzyme (Figure 2-6B) (Xie, 2009).

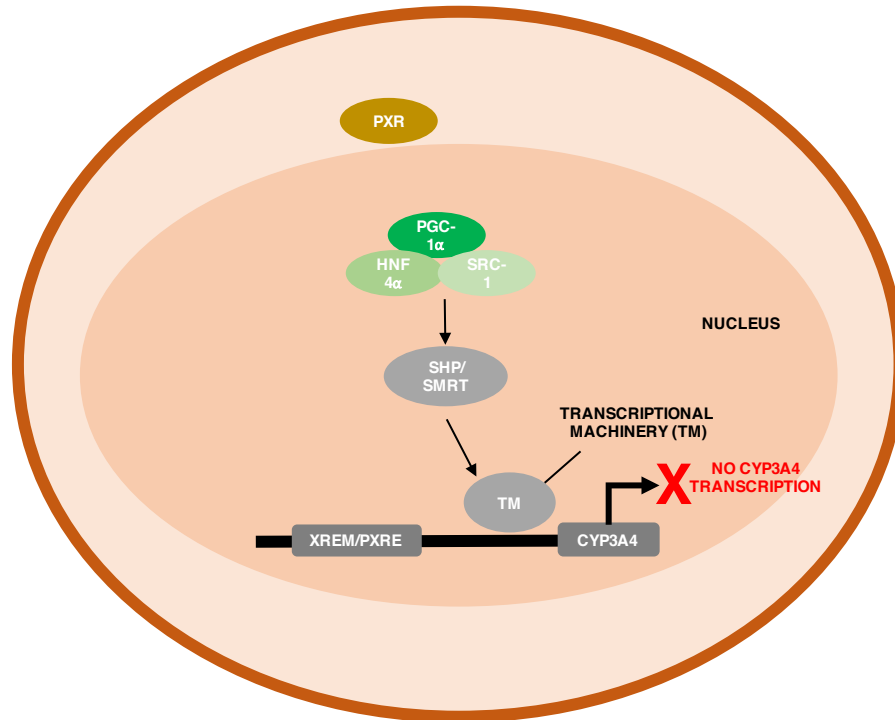
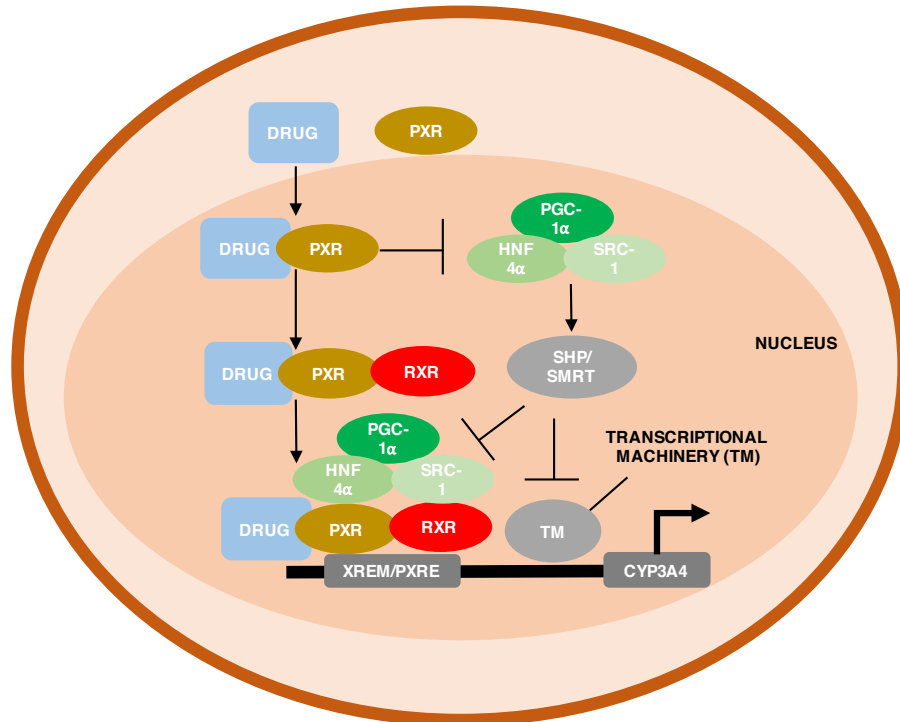
A**B**

Figure 2- 6 Graphical representation of the activation of PXR nuclear receptor by a xenobiotic (drug), resulting in the expression of CYP in presence of coactivators. **A**, in absence of a ligand, the orphan nuclear receptors small heterodimer partner (SHP) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) are transcribed in presence of the coactivators steroid receptor coactivator-1 (SRC-1), hepatocyte nuclear factor α (HNF4 α) and peroxisome proliferator –activated receptor γ coactivator-1 α (PGC-1 α). As a result, no CYP transcription will occur. **B**, in presence of a ligand (drug), the PXR receptor translocates to the nucleus of the cell and the ligand-PXR complex will form a heterodimer with RXR. CYP transcription will initiate upon the binding of the complex coactivators (SRC-1, HNF4 α and PGC-1 α)-ligand-PXR-RXR complex will bind to the xenobiotic responsive enhancer molecule (XREM)/PXRE region.

2.4.4.2 Experiment protocol

Four RENISUS plant extracts (*Erythrina mulungu* Benth., *Cordia verbenaceae* A. DC., *Solanum paniculatum* L. and *Lippia sidoides* Cham.) were subject to hPXR activation assays. The experiment protocol for hPXR activation assay required first the adding of a luciferase reporter gene, which is a downstream of the PXRE. Once the ligand binds to the PXRE, the amount of luciferase enzyme is quantified by the addition of luciferin substrate and detection after cell lysis.

After 24 hours seeding, HeLa cells (4×10^4 cells/well) were transiently co-transfected with 60ng of pM-Gal4-PXR-LBD and 240ng of Gal4 luciferase reporter using lipofectamine 2000 reagent according to the manufacturer's protocol. Transfected cells were treated with increasing concentrations of plant extract and/or rifampicin $1\mu\text{M}$ (EC_{50}). Luciferase activity was measured after 24 hours, according to the manufacturer's protocol in a 20/20n Glomax luminometer and reported as a response (%) compared to cells treated only with RIF.

2.4.5 Intracellular GSH assay

2.4.5.1 Background

GSH is a ubiquitous and unusual tripeptide thiol (L-γ-glutamyl-L-cysteinylglycine) or α-amino acid found in the cortex, medulla, cytosol, mitochondria, molecules, blood and most frequently in the liver of various mammalian cells in millimolar range (0.5-10mM). It is known to function either directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism and protection of cells (Meister and Anderson, 1983). Out of all the functions of GSH, two are relevant in the process of cell detoxification. They are: (1) its role as an antioxidant by removing potentially toxic electrophilic compounds such as free radicals, peroxides, and alkylating agent (Ionescu and Caira, 2005, Allen et al., 2000) and (2) its role in phase II metabolism of xenobiotics. In general, both roles relate to the general function of GSH to protect cells against oxygen toxicity (Fahey and Sundquist, 2006).

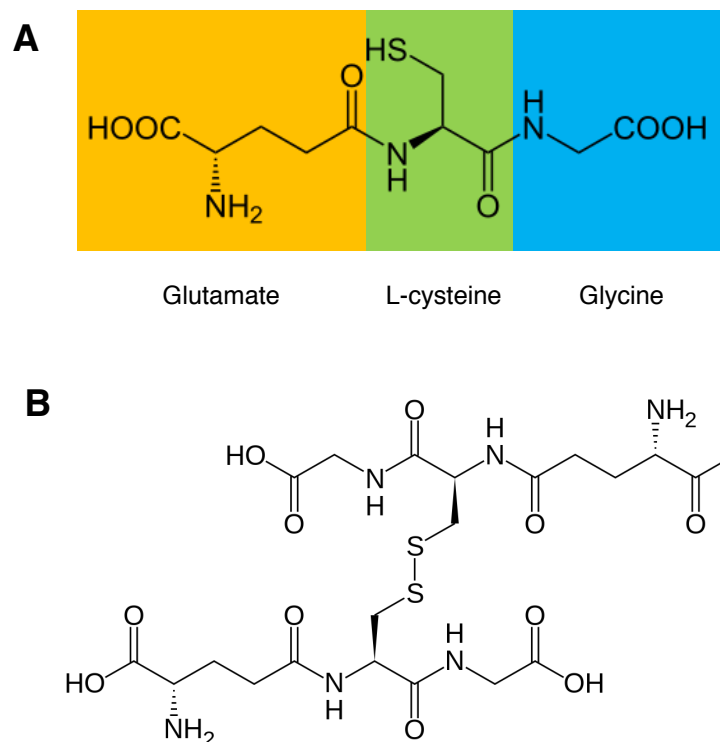


Figure 2- 7 Chemical structures of the reduced GSH (A) and oxidized GSH (GSSG) (B). The tripeptide is formed by the amino acids glutamate, L-cysteine, and glycine.

GSH exists in two different forms within cells: the reduced sulfhydryl form (GSH) and the glutathione disulphide form (GSSG) (Figure 2-7). In healthy cells, GSSG is less than 0.2% of the total GSH concentration. Oxidative stress has a profound effect on the cellular thiol balance and can lead to a decreased GSH/GSSG ratio in many body organs. Reactive oxygen species (O_2^- , OH^- , H_2O_2 and $R-OOH$) as well as xenobiotics are neutralised by GSH through a cascade of detoxification mechanisms involving the enzymes glutathione peroxidases (GPx), GST and GR (Rahman et al., 2007).

GSH synthesis occurs in the cytosol through a two-step ATP-requiring enzymatic process. First, γ -glutamylcysteine is formed by the conjugation of L-cysteine and glutamate. This step is catalysed by the γ -glutamylcysteine

synthase (Figure 2-8A). The second step is catalysed by the enzyme GSH synthase. The previous formed compound γ -glutamylcysteine conjugates with the amino acid glycine to generate γ -glutamylcysteinylglycine, or GSH (Figure 2-8B) (Lu, 2013) .

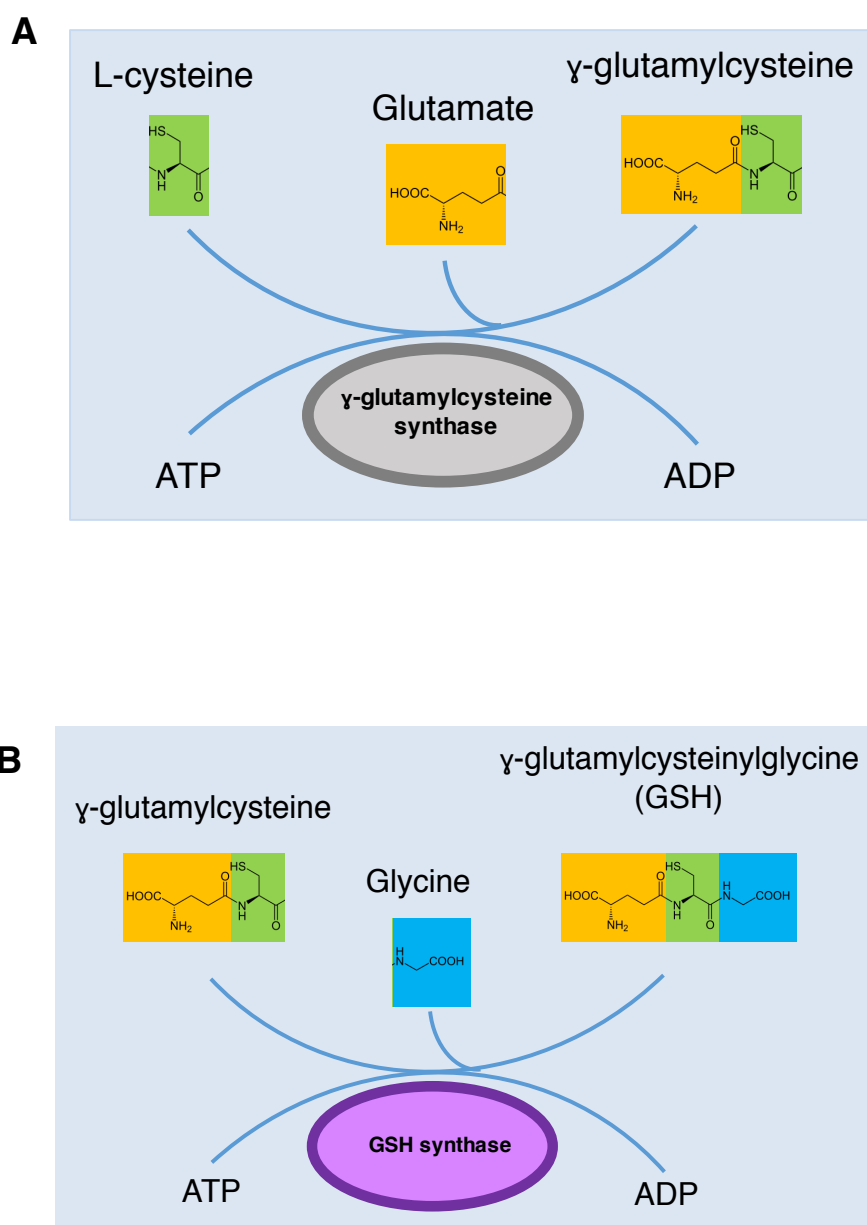


Figure 2- 8 The two-step intracellular GSH synthesis. A, γ -glutamylcysteine is formed by the conjugation of L-cysteine and glutamate, catalysed by γ -glutamylcysteine synthase. B, γ -glutamylcysteine conjugates with glycine to generate γ -glutamylcysteinylglycine (GSH). This step is catalysed by GSH synthase.

Conjugation with GSH avoids the reaction of electrophilic compounds to nucleophilic ones in macromolecules such as proteins and nucleic acids. When the conjugate is formed, it has to undergo further metabolic reactions in order to form mercapturic acid, which has a transport, binding and scavenger function whereby transferases is alkylated by the action of reactive intermediates (protective and detoxification role). The final product is then eliminated from the organism (Sies and Ketterer, 1988).

The thioether (GSH conjugate) undergoes enzymatic reactions and these mechanisms are catalysed by the enzyme GGT which hydrolyses the glutathione-S-conjugate at the γ -glutamyl bond. GGT enzyme (transpeptidase) removes glutamate from the conjugate yielding a dipeptide, which is subsequently attacked by the second GGT enzyme (peptidase) to remove the amino acid glycine. The cysteine conjugate formed then undergoes then N-acetylation reaction by N-acetyltransferase (NAT) yielding N-acetylcysteine conjugate. Acetylation is a mechanism in which an acetyl group ($\text{CH}_3\text{-COO}^-$) is transferred to a molecule (in this case, an acetylation of amine group) by N-acetylase. This is a “ping-pong” mechanism made in two steps where the N-acetylase catalyses the transfer of the acetyl group from acetyl-CoA to the GSH conjugate and forms an acetyl-enzyme that will then undergo the acetylation of the arylamine with regeneration of the enzyme (Figure 2-10) (Ionescu and Caira, 2005). This detoxification pathway can also be detected *in vitro* by using HepG2 cells as model system (Rebbeor et al., 1998).

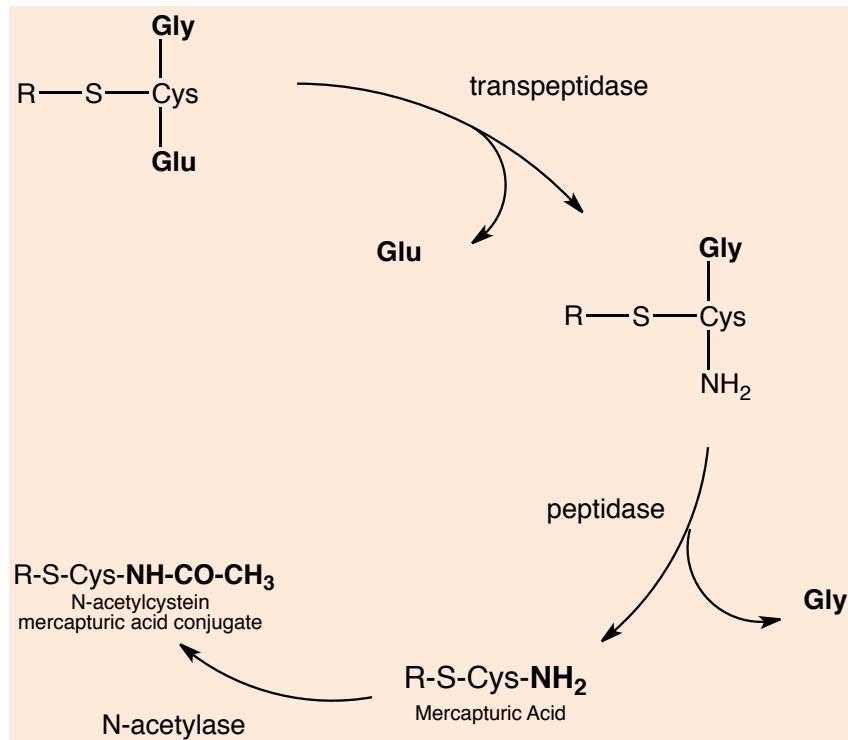


Figure 2- 9 Formation of mercapturic acid conjugate from a GSH conjugate. After the conjugation of GSH with the metabolite, the complex is released from the cell where is firstly borken down by a transpeptidase (γ-glutamyl transferase) followed by the release of the amino acid glutamate. The remaining part of the complex is broken down once again by a dipeptidase, which will dissociate the amino acid glycine from the rest of the molecule to form the mercapturic acid. In order to improve the polarity of the mercapturic acid, an acetyl group is added to the molecule to form the N-acetylcystein mercapturic acid conjugate, which will be further excreted in the urine.

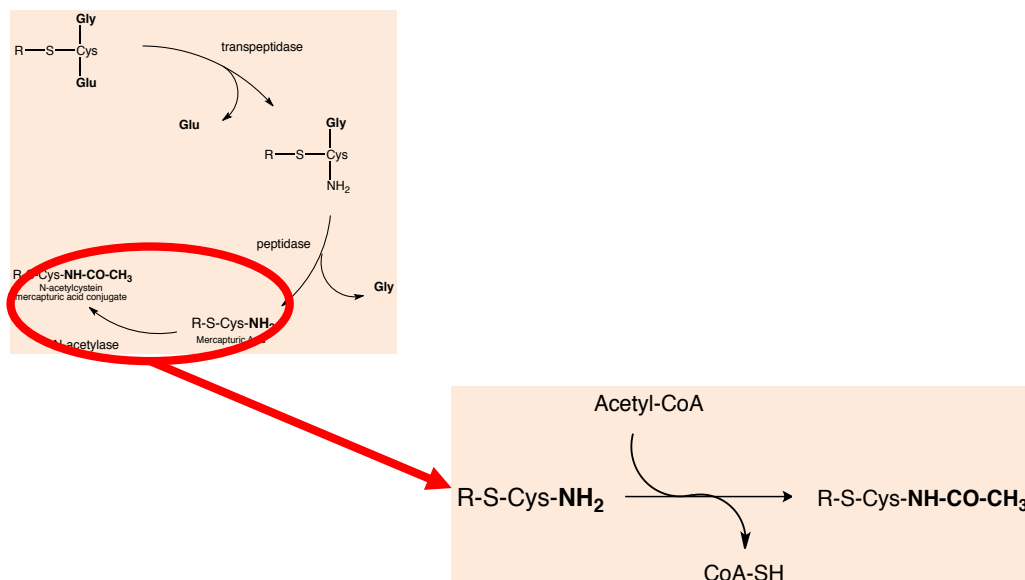


Figure 2- 10 N-acetylation of mercapturic acid.

Disease conditions or xenobiotics that induce oxidative stress, in turn affect intracellular GSH concentrations. By measuring the intracellular GSH levels in the presence of the plant extracts, we can determine if the extracts will enhance or diminish the detoxification effects of GSH. Several protocols for measurement of intracellular GSH levels have been published using chemical, enzymatic, high-pressure chromatographic, and flow cytometric methods. However, issues including low throughput caused by a complicated and time-consuming sample preparation and the use of large amounts of samples made it necessary to develop a microassay, which uses a 96-well microtiter plate to measure GSH levels in HepG2 cells (Allen et al., 2000). The method used in the intracellular determination of GSH levels was adapted from that described by Allen and coworkers (2000) and Rahman and coworkers (2007) with slight modifications (Allen et al., 2000, Rahman et al., 2007).

The assay was based on the reaction of GSH with DTNB (5, 5'-dithio-bis (2-nitrobenzoic acid), also known as Ellman's reagent to produce TNB (5-thio-2-nitrobenzoic acid), a yellow coloured chromophore which can be measured spectrophotometrically at 405nm, and an oxidized GSH-TNB adduct (GS-TNB). The GS-TNB adduct was reduced by GR in the presence of NADPH to form GSH and TNB, with the former being recycled back into the system to produce more TNB (Figure 2-11) (Rahman et al., 2007).

The kinetic reaction is similar to an S_N2 mechanism. The nucleophile (GSH anion - GS^-) attacks the disulphide bridge of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) in order to form the monothiol anion TNB that is highly coloured, which has a maximal absorbance at 412nm. The absorbance of TNB formed corresponds to the amount of intracellular GSH in the sample. The enzyme GR in presence of NADPH will reduce the disulphide product (GS-TNB), therefore recycling therefore GSH back into the reaction (Szajewski and Whitesides, 1980, Allen et al., 2000, Rahman et al., 2007).

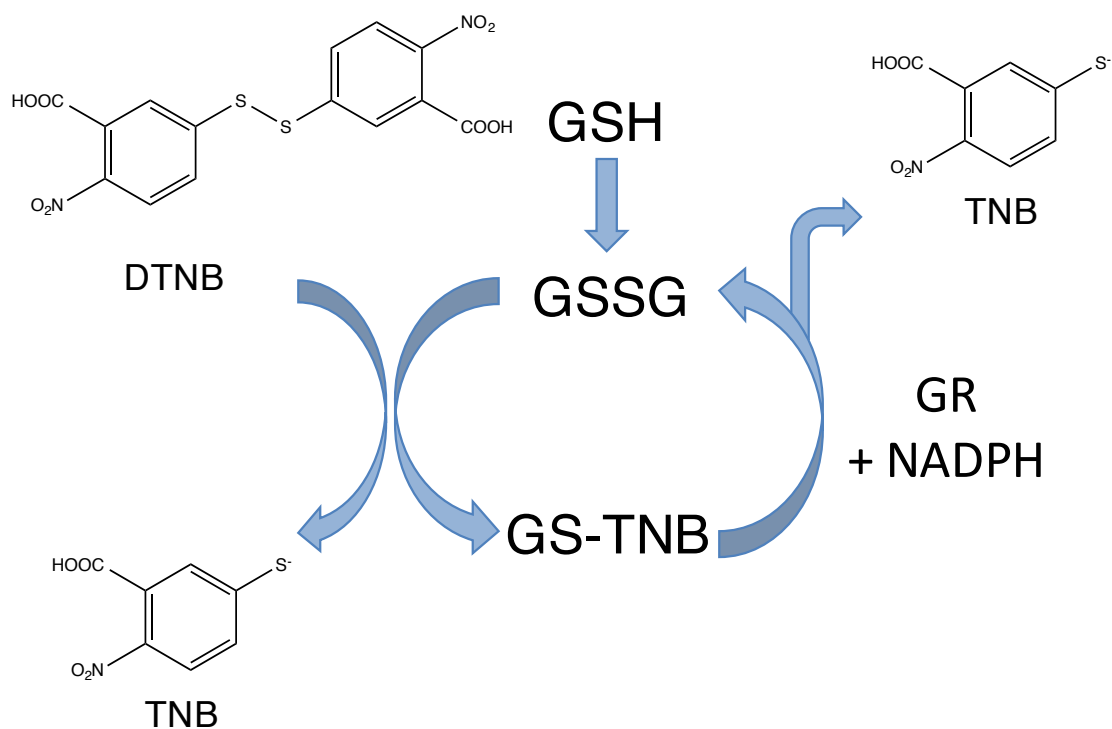


Figure 2- 11 Enzymatic recycling of GSH from glutathione disulphide (GSSG) by glutathione reductase (GR) in the presence of NADPH. GSH reacts with DTNB to GS-TNB and TNB, with subsequent reduction of the former to GSH. TNB can be measured at 405 nm. (Adapted from Rahman et al., 2007).

The GSSG present in the sample is also reduced by GR to form two molecules of GSH. Thus, the amount of GSH measured represented the sum of reduced and oxidised GSH in the sample. The rate of change in absorbance of TNB at 405nm is linearly proportional to the total concentration of GSH in the sample. The concentration of an unknown sample can therefore be determined by calculating from the linear equation or the regression curve generated from several standard concentrations of GSH.

2.4.5.2 Experiment protocol

HepG2 cells (4×10^4 cells per well) were seeded out in sterile, flat bottom 96-well plates and incubated overnight to allow for cell attachment. The next day, the cells were treated with the plant extracts ($100\mu\text{g/mL}$) to be tested in triplicates. Three wells were not treated with any drug and served as the media control, while three other wells were treated with buthionine sulfoximine (BSO) ($10\mu\text{M}$) as the positive control. All drug/extract incubations were done for 24 hours.

Prior to commencing the GSH assay, the following solutions were prepared: phosphate buffer solution containing 168mL of a 286mM solution of sodium phosphate dibasic in distilled water, 32mL of a 286mM solution of sodium phosphate monobasic and 6.3mM EDTA; 5% SSA in double distilled water; 0.1% Triton X-100; 1mM stock solution of reduced glutathione in 5% SSA solution; and 10mM DTNB in phosphate buffer solution. Apart from the DTNB, all the other solutions were able to be stored in the fridge at 2 - 4 °C for up to a week. 2.39mM or 2mg/mL β -NADPH in phosphate buffer solution was freshly prepared on the day of the assay.

24 hours after incubation of the extract/drug, the cells were washed with PBS ($200\mu\text{L}$ per well) and the PBS was aspirated off. 0.1% Triton X-100 ($60\mu\text{L}$) was added to each well of the plate containing cells and then shaken on a microplate shaker for 2 minutes to lyse the cells. The UV light of the laminar flow hood was turned off for the rest of the assay to minimise GSH oxidation.

Subsequently, 5% SSA solution ($25\mu\text{L}$) was added and the plate shaken for another 2 minutes. After shaking, the lysed cell solution ($25\mu\text{L}$) was transferred to the wells of the next three inner columns of the plate. 5% SSA solution ($25\mu\text{L}$) was added to each well of the first column of the plate as a blank. A double dilution of a standard solution of reduced glutathione in 5% SSA ($25\mu\text{L}$), starting from $100\mu\text{M}$ GSH as the highest concentration was then prepared.

The GSH reaction buffer needed for the assay was prepared by adding the freshly prepared β -NADPH (1mL) and GR ($3\mu\text{L}$) (equivalent to 2 units of GR) to the phosphate buffer solution (7.5mL) for each 96-well plate. This was

vortexed for 5 seconds and then the DTNB solution (1mL) was immediately thawed and added to the reaction buffer and then vortexed for another 5 minutes. All solutions to be used were kept on ice until needed. The reaction buffer (125 μ L) was added to each well of the 96-well plate containing either the blank SSA solution (25 μ L), the GSH standards (25 μ L) or the cell lysate solution (25 μ L). The microplate was immediately covered and then wrapped in foil paper and then shaken in the microplate for 15 seconds. Immediately after, the absorbance of the wells of the microplate was read in a kinetic cycle in the TECAN plate reader every 30 seconds for 5 minutes at 405nm (11 readings). The slope of the curve obtained from the 11 readings of each of the GSH standard concentrations (otherwise known as the I-slope), which represents the rate of change of GSH levels at that concentration plotted against the GSH concentration to give a regression curve (otherwise known as the F-slope curve). The concentration of GSH in the samples from the cell lysate solutions was then estimated by substituting the average I-slope values for each of the extract/drug into the equation of the F-slope curve (Ezuruike, 2015).

2.4.6 GGT activity assay

2.4.6.1 Background

GGT is a membrane-localised enzyme that plays a key role in the maintenance of GSH at normal levels in the body. The enzyme provides the amino acid cysteine, which is the precursor for GSH synthesis. As GSH cannot be hydrolysed by general peptidases due to its unusual peptide bond linking glutamate and cysteine through the γ -carboxyl group of glutamate instead of the conventional α -carboxyl group, GGT has the crucial function of breaking down that bond. The enzyme catalyses the cleavage of the γ -glutamyl bond of GSH and the transfer of the γ -glutamyl group to water, amino acids or peptides.

The amino acid cysteine is fundamental for GSH synthesis in the cell. In this cycle, GSH is transported out of the cell and it is first broken-down by GGT, form γ -glutamyl amino acid and cysteinylglycine. γ -glutamyl amino acid is transported back into the cell and metabolised to release the amino acid and 5-

oxoproline, which will then be further converted to glutamate and reincorporated to GSH. Cysteinylglycine is cleaved by dipeptidases to produce glycine and cysteine, which is transported back into the cell. The majority of cysteine uptake by the cell is to synthesise GSH and the remaining part is used for the synthesis of proteins and/or broken down into sulphate and taurine (Figure 2-12). When GGT activity is deficient, GSH molecule is not cleaved and, as a consequence, the cells are unable to re-uptake the cysteine for the tripeptide synthesis.

In order to conduct our GGT activity enzymatic assay we used HepG2 cells, which displays a relatively high activity of the enzyme. Also, GGT activity in HepG2 cells are comparable to that seen in human liver and higher than the activity found in rat liver (Rebbeor et al., 1998). The GGT enzyme activity assay was performed using GpNA as it consists of the release of *p*-nitroaniline, which was monitored by spectrophotometer at 405nm. The GGT catalyses the transfers of the γ -glutamyl group from the substrate GpNA (colourless) to the acceptor glycylglycine (gly-gly), liberating the chromogen *p*-nitroaniline, which is proportional to the GGT present.

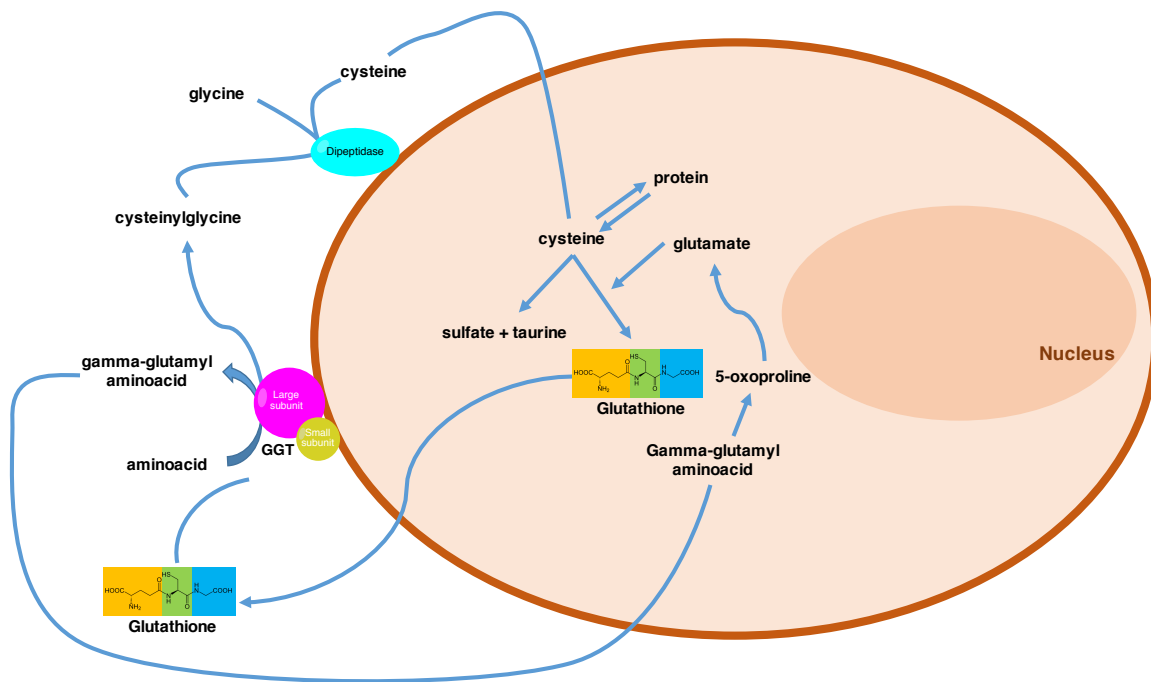


Figure 2- 12 The γ -glutamyl cycle. When GSH is transported out of the cell, the enzyme gamma-glutamyl transferase (GGT) will break down the tripeptide and transfer the γ -glutamyl moiety to an amino acid, forming γ -glutamyl amino acid and cysteinyglycine. The γ -glutamyl amino acid is transported back to the cell where it will be metabolized to release the amino acid and 5-oxoproline, which will be converted to glutamate and reincorporated into GSH. Dipeptidase will break down cysteinyglycine into glycine and cysteine. The latter will be transported back to the cell to be reincorporated into GSH. The amount of cysteine which is not used for GSH synthesis will be incorporated into newly synthesized proteins and/or broken down into sulphate and taurine.

2.4.6.2 Experiment protocol

GGT activity assay was conducted according Rebbeor and coworkers (1998) with slight modifications. Briefly, after 24 hours of incubation of HepG2 cells (1×10^6 cells/well) with the GGT inhibitor acivicin ($5\mu\text{M}$) or plant extracts ($100\mu\text{g/mL}$), media was aspirated and cells were washed with PBS. 4mL of pre-warmed Gly-Gly buffer (115mM Tris, 138mM Gly-Gly) and 400 μL of the substrate GpNA (29.6mg/mL of HCl 0.5mmol/L) were added to the wells and plates were incubated for 10 minutes. Subsequently, 500 μL of the content of each well were transferred to 24-well plates and absorbance was measured in the plate reader (405nm). The absorbances were converted into absolute amounts by means of a calibration line using p-nitroaniline (Rebbeor et al., 1998).

2.4.7 Modulation of P-glycoprotein dependant Rh-123 efflux in Caco-2 cells

2.4.7.1 Background

Rh-123 is a cationic hydrophilic fluorescent P-gp substrate, which has been used as a probe to assess the functional activity of P-gp in a variety of cell lines and assays (Lee et al., 1994). In the intestine, the absorptive transport of Rh-123 occurs primarily by a passive diffusion process via the paracellular pathway. However after absorption, Rh-123 is secreted back into the lumen, first of all via a transcellular uptake process through the basolateral membrane followed by P-gp mediated efflux through the apical membrane (Troutman and Thakker, 2003).

Caco-2 VCR cells were used for this drug accumulation/efflux assay where the amount of the Rh-123 retained by the cells after an incubation period (in the presence or absence of inhibitors) was measured spectrofluorometrically (Chieli and Romiti, 2008). Compounds that interact with the P-gp transporter either as substrates or inhibitors as depicted in figure 2-13 modulate the P-gp mediated efflux of Rh-123 and could therefore be identified.

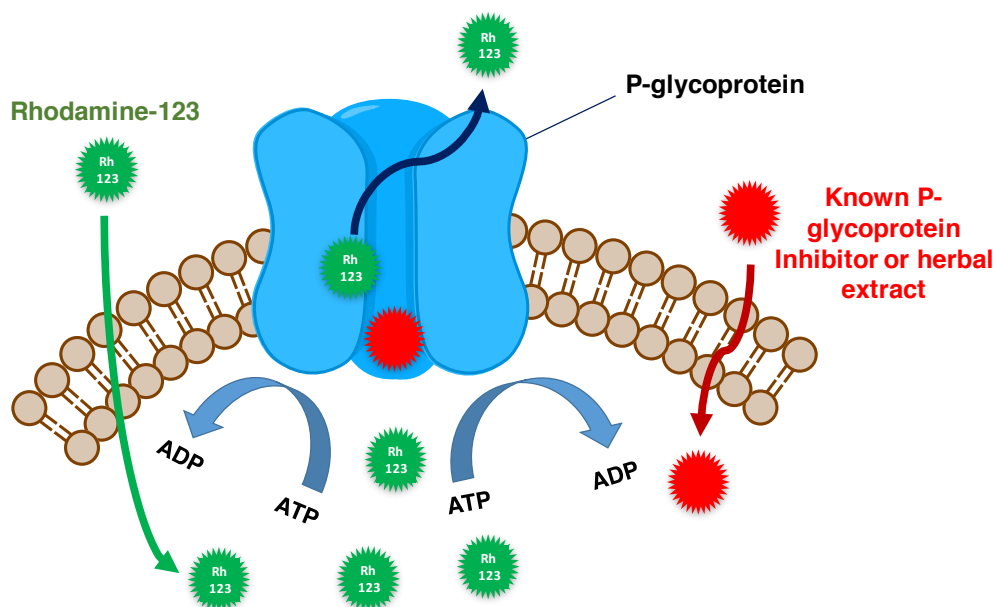


Figure 2- 13 Schematic representation of the inhibition of Rh-123 efflux by modulators of the P-glycoprotein transporter. Rh-123 is a fluorescent dye and a P-gp substrate. Upon cell uptake, Rh-123 will be effluxed out by P-gp in an ATP-requiring process. In presence of a p-glycoprotein inhibitor, the efflux is reduced allowing the accumulation of Rh-123 in the cell, which is detected and quantified by a spectrofluorometer.

2.4.7.2 Experiment protocol

The protocol used for this assay is as described in Chieli and coworkers (2009), with slight modifications (Chieli et al., 2009). Caco-2 VCR cells to be used were routinely grown in the appropriate complete growth media in 75cm² tissue culture flasks as described in section 2.4.1.3. The culture media of the cells were usually changed a day before plating out to ensure that the cells were in their exponential stage of growth. The media of Caco-2 VCR cells was replaced with complete media without vincristine at this point.

80 to 90% confluent culture flask of cells were trypsinised and then plated out in the inner wells of a sterile 96-well plate at a density of 1.5×10^4 cells/well in full culture media (without vincristine). The cells were then placed in the incubator overnight to allow for attachment. The next day, the growth media of the cells was changed to serum free media (without 10% FBS). This was to ensure a more uniform condition for the majority of the cell population and to prevent adsorption of drugs to the serum proteins. The microplates were then replaced back in the incubator for 24 hours.

On the day of the assay, Rh-123 (5µg/mL) solution in serum free media sufficient for the assay was prepared from a stock solution in DMSO (5mg/mL) (solution A). All the extracts to be tested were then prepared (100µg/mL) in solution A (solution B), while 20µM verapamil (Securon® I.V. 2.5mg/mL, Abbot Laboratories Limited) was also prepared in solution A as positive control (solution C). The media in each of the wells of the microplate was then aspirated off and then replaced with either solution A, B or C in replicates. The microplates were placed back in the incubator for 2 hours to allow for intracellular Rh-123 accumulation within the cells. During this time, 20µM verapamil in PBS (solution D) was also prepared and stored in the fridge at 4°C.

After the two hours of incubation, the media in the wells were all aspirated off and each well was washed three times, gently but quickly with the ice cold solution D (200µL) to stop the P-gp efflux activity. 0.1% Triton X-100 in PBS (100µL) was then added to each well and then placed in the incubator for 15 minutes to lyse the cells and completely solubilise the intracellular Rh-123. Cell lysates (80µL) were transferred to a 96-well black plate and the

fluorescence intensity of each well was measured in a Tecan® plate reader (Exc-485nm, Em-525nm). The cellular accumulation of Rh-123 for each of the extracts was expressed as the percentage of the accumulation measured for Rh-123 only (solution A), that is under control conditions.

2.4.8 Statistical Analysis

Collected data were analysed as means \pm SD of at least three independent experiments. Statistical significance was measured by student *t*-test and one-way ANOVA followed by Bonferroni correction using GraphPad InStat (GraphPad Software Inc., La Jolla, CA, USA). Results with a value of $p < 0.05$ were considered significant.

3 RESULTS

3.1 AUTHORISATION FOR ACCESSING THE BRAZILIAN GENETIC HERITAGE COMPONENT FROM CGEN AND YIELD OF PLANT EXTRACTS

The authorisation from CGEN for the initiation of our research with the Brazilian plant extracts was issued in May 2014 (Appendix 7.4). A total number of twenty-four plant samples comprising twenty-three species from nineteen families were collected from either the “Farmácias vivas” or bought from the herbal suppliers in Brazil in 2014.

All samples were extracted by infusing with hot water. The yields of the different plant samples varied from as low as 5.03% w/w (*Artemisia absinthium*) to as high as over 14.95% w/w (*Cordia verbenaceae*) (Table 3-1).

Table 3- 1 Tabular summary of plant samples - Collection & Extraction

Plant name	Family	Local name	Plant Part	Sample ID	Place of collection/purchase	Extraction method	Yield (%w/w)
<i>Apuleia ferrea</i> (Mart.) Baill.	<i>Leguminosae</i>	Pau-ferro	Stem-bark	AF1	Florien	Infusion	14.18
<i>Artemisia absinthium</i> L.	<i>Compositae</i>	Losna	Herb	AA1	Florien	Infusion	5.03
<i>Bidens pilosa</i> L.	<i>Compositae</i>	Picão	Leaves	BP1	Florien	Infusion	6.44
<i>Casearia sylvestris</i> Sw.	<i>Salicaceae</i>	Guaçatonga	Aerial parts	CAS	Florien	Infusion	7.52
<i>Cordia verbenaceae</i> A. DC.	<i>Boraginaceae</i>	Erva-baleeira	Leaves	CV1	Farmácia Viva	Infusion	14.95
<i>Costus spicatus</i> (Jacq.) Sw.	<i>Costaceae</i>	Cana-de-macaco	Leaves	CS1	Florien	Infusion	9.58
<i>Equisetum arvense</i> L.	<i>Equisetaceae</i>	Cavalinha	Herb	EA1	Florien	Infusion	8.93
<i>Erythrina mulungu</i> Benth.	<i>Leguminosae</i>	Mulungu	Stem-bark	EM1	Santos Flora	Infusion	5.77
<i>Lamium album</i> L.	<i>Lamiaceae</i>	Urtiga-branca	Herb	LA1	Florien	Infusion	7.65
<i>Lippia sidoides</i> Cham.	<i>Verbenaceae</i>	Alecrim-pimenta	Leaves	LS1	Farmácia Viva	Infusion	13.33
<i>Malva sylvestris</i> L.	<i>Malvaceae</i>	Malva	Flower	MSF	Florien	Infusion	6.16
			Leaves	MSH	Florien	Infusion	11.87
<i>Maytenus ilicifolia</i> Mart. ex Reissek	<i>Celastraceae</i>	Espinheira-santa	Leaves	MI1	Florien	Infusion	8.49
<i>Morus nigra</i> L.	<i>Moraceae</i>	Amoreira-preta	Leaves	MN1	Florien	Infusion	10.68
<i>Passiflora incarnata</i> L.	<i>Passifloraceae</i>	Maracujá	Leaves	PI1	Florien	Infusion	9.13
<i>Persea americana</i> Mill.	<i>Lauraceae</i>	Abacateiro	Leaves	PA1	Florien	Infusion	7.19
<i>Rhamnus purshiana</i> DC.	<i>Rhamnaceae</i>	Cáscara-sagrada	Stem-bark	RP1	Santos Flora	Infusion	12.33
<i>Salix alba</i> L.	<i>Salicaceae</i>	Salgueiro-branco	Leaves	SA1	Florien	Infusion	12.51
<i>Schinus terebinthifolia</i> Raddi	<i>Anacardiaceae</i>	Aroeira	Stem-bark	ST1	Florien	Infusion	11.49
<i>Solanum paniculatum</i> L.	<i>Solanaceae</i>	Jurubeba	Aerial parts	SP1	Santos Flora	Infusion	9.27
<i>Solidago microglossa</i> DC.	<i>Compositae</i>	Arnica brasileira	Aerial parts	SM1	Santos Flora	Infusion	13.43
<i>Syzygium jambolanum</i> (Lam.) DC.	<i>Myrtaceae</i>	Jambolão	Leaves	SJ1	Santos Flora	Infusion	10.05
<i>Tabebuia avellanedae</i> Lorentz ex Griseb.	<i>Bignoniaceae</i>	Ipê	Stem-bark	TA1	Santos Flora	Infusion	13.10
<i>Vernonia polyanthes</i> Less.	<i>Compositae</i>	Assa-peixe	Aerial parts	VP1	Florien	Infusion	14.72

3.2 PHYTOCHEMICAL ANALYSIS

HPTLC analysis in the four RENISUS Brazilian native plants showed that *Cordia verbenaceae* A. DC. contains caffeic acid at $R_f = 0.87$ and chlorogenic acid at $R_f = 0.38$. *Solanum paniculatum* L. contains gallic acid at $R_f = 0.81$, rutin at $R_f = 0.20$ and chlorogenic acid at $R_f = 0.38$. *Lippia sidoides* Cham. contains luteolin at $R_f = 0.86$ and minor amounts of quercetin at $R_f = 0.90$. *Erythrina mulungu* Benth. did not contain any of these metabolites in significant amounts (Figures 3-1, 3-2 and 3-3 and table 3-2).

HPLC fingerprinting analysis targeting phenolic compounds showed the presence of tannins, chlorogenic acid, caffeic acid, rutin, phenolic acids and glycosylated flavonoids in most of the samples (Table 3-2 and Appendix 7.5).

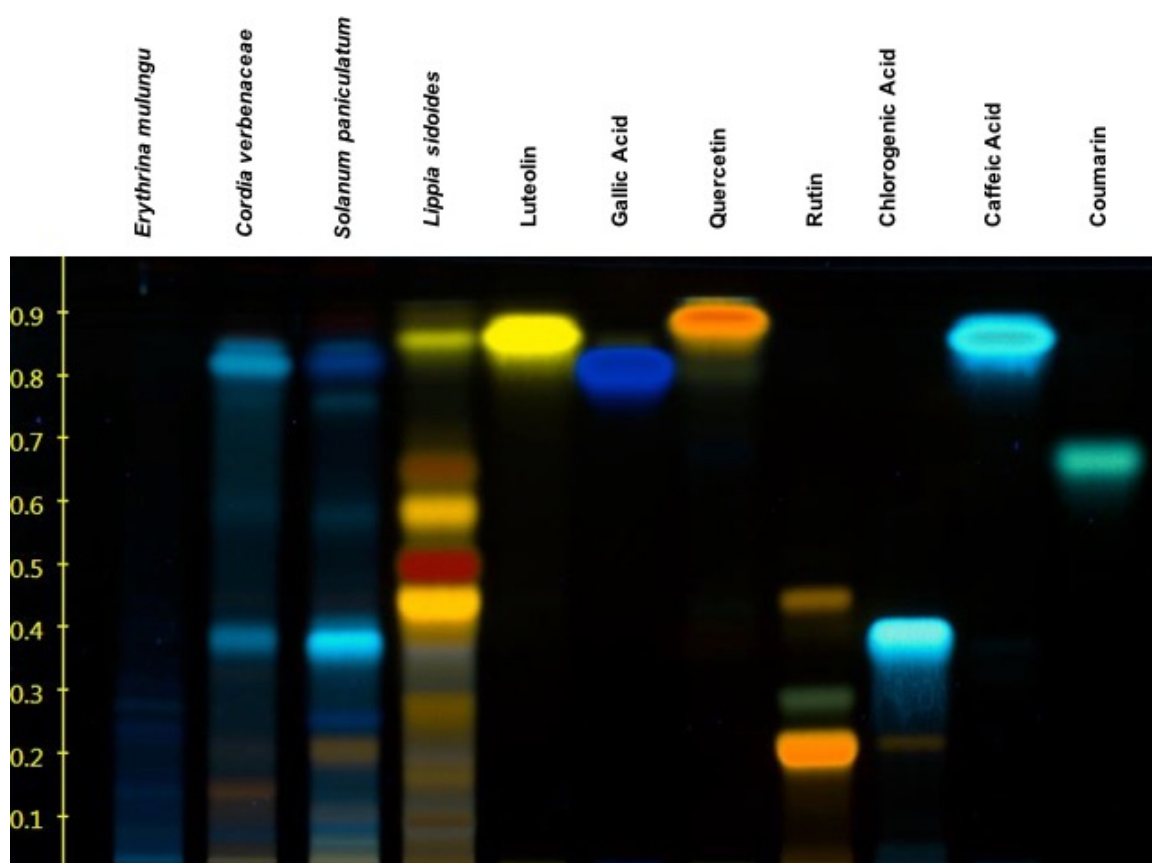


Figure 3- 1 365nm after derivatization.

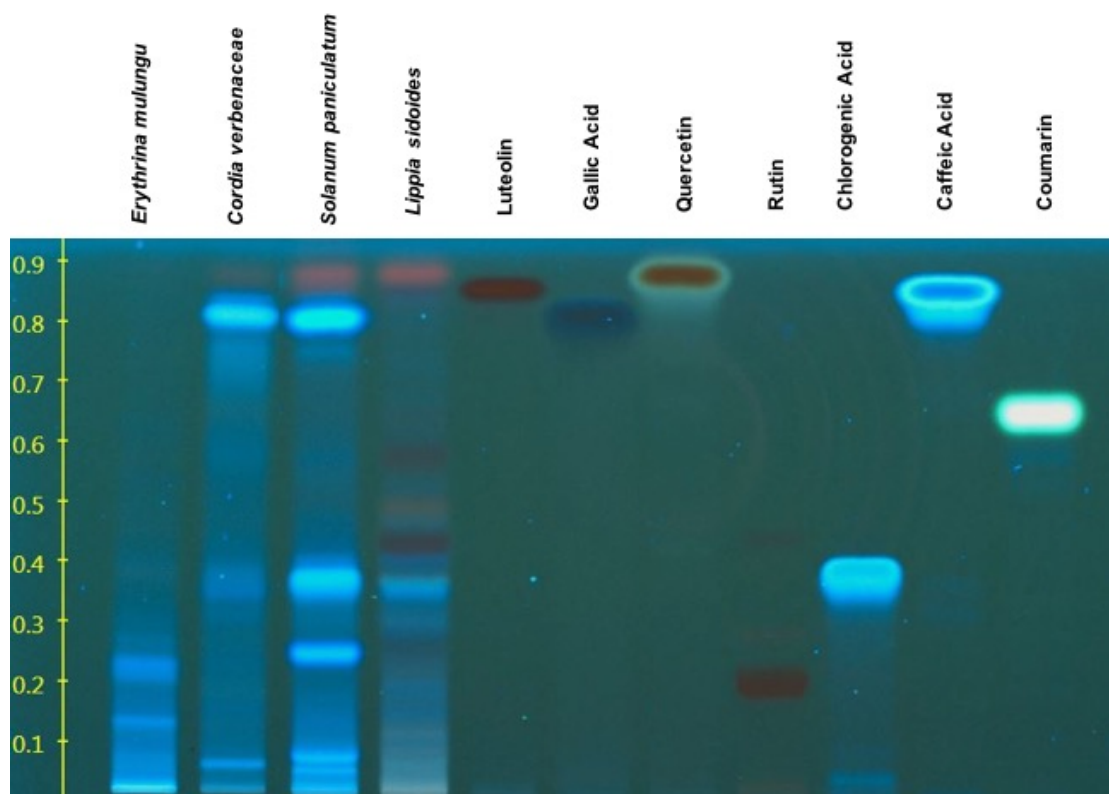


Figure 3- 3 365nm before derivatization.

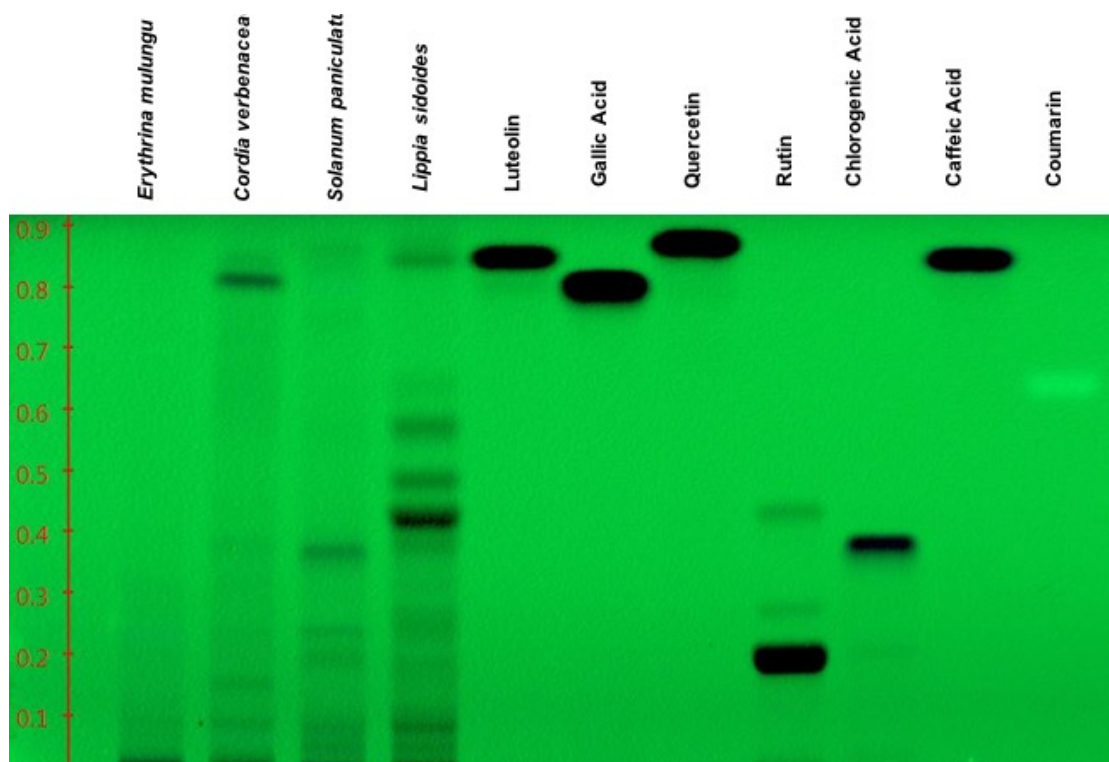


Figure 3- 2 235nm after derivatization.

Table 3- 2 Tabular summary of phenolic compounds present in plant samples detected by HPTLC and HPLC-DAD.

Plant name	Phenolic standards detected in sample based on HPTLC* and HPLC-DAD* analysis
<i>Apuleia ferrea</i> (Mart.) Baill.	Tannins*
<i>Artemisia absinthium</i> L.	Chlorogenic acid*
<i>Bidens pilosa</i> L.	Chlorogenic acid*, Caffeic acid* and flavonoids*
<i>Casearia silvestris</i> Sw.	Chlorogenic acid* and phenolic acids*
<i>Cordia verbenaceae</i> A. DC.	Caffeic acid*, chlorogenic acid*, phenolic acids*
<i>Costus spicatus</i> (Jacq.) Sw.	Glycosylated flavonoids* and quercetin*
<i>Equisetum arvense</i> L.	Caffeic acid acid*, phenolic acids* and glycosylated flavonoids*
<i>Erythrina mulungu</i> Benth.	None*, tannins*, phenolic acids*, glycosylated flavonoids*
<i>Lamium album</i> L.	Tannins*, chlorogenic acid*, glycosylated flavonoids*, phenolic acids*
<i>Lippia sidoides</i> Cham.	Luteolin*, quercetin*, flavonoids*
<i>Malva sylvestris</i> L. (MSH and MSF)	Phenolic acids* and flavonoids*
<i>Maytenus ilicifolia</i> Mart. ex Reissek	Glycosylated flavonoids*
<i>Morus nigra</i> L.	Chlorogenic acid*
<i>Passiflora incarnata</i> L.	Glycosylated flavonoids*
<i>Persea americana</i> Mill.	Chlorogenic acid* and Glycosylated flavonoids*
<i>Rhamnus purshiana</i> DC.	Chlorogenic acid* and Glycosylated flavonoids*
<i>Salix alba</i> L.	None*
<i>Schinus terebinthifolia</i> Raddi	Phenolic acids*
<i>Solanum paniculatum</i> L.	Gallic acid*, rutin*, chlorogenic acid* and Glycosylated flavonoids*
<i>Solidago microglossa</i> DC.	Chlorogenic acid* and Glycosylated flavonoids*
<i>Syzygium jambolanum</i> (Lam.) DC.	Glycosylated flavonoids*
<i>Tabebuia avellaneda</i> Lorentz ex Griseb.	Tannins*, phenolic acids*
<i>Vernonia polyanthes</i> Less.	Glycosylated flavonoids*

3.3 SRB, NEUTRAL RED AND MTT ASSAYS FOR DETERMINATION OF CELL VIABILITY/CYTOTOXICITY

The three selected cell viability assays were conducted after 24 hours incubation with either the plant extracts or drugs. Viability of HepG2, Caco-2 VCR and HeLa was assessed prior to evaluation of the extracts using ethanol and hydrogen peroxide (H₂O₂) as positive references (Figure 3-4). As all the extracts were completely dissolved in water, no DMSO was used in the samples.

The maximum non-toxic concentration (MNTC) values were determined for all the extracts using HepG2 and Caco-2 VCR cell lines. The MNTC of *Erythrina mulungu* Benth, *Cordia verbenaceae* A. DC, *Solanum paniculatum* L. and *Lippia sidoides* Cham. were also assessed in HeLa cells. Initially, the experiment was conducted using concentrations of extracts ranging from 100 to 1000 µg/mL. However, no toxicity was found in concentrations above 100 µg/mL (viability ≥ 80%) (Table 3-3). Therefore, our preclinical study was conducted using 100 µg/mL of each extract, which is considered a higher limit than necessary to get physiological relevant results.

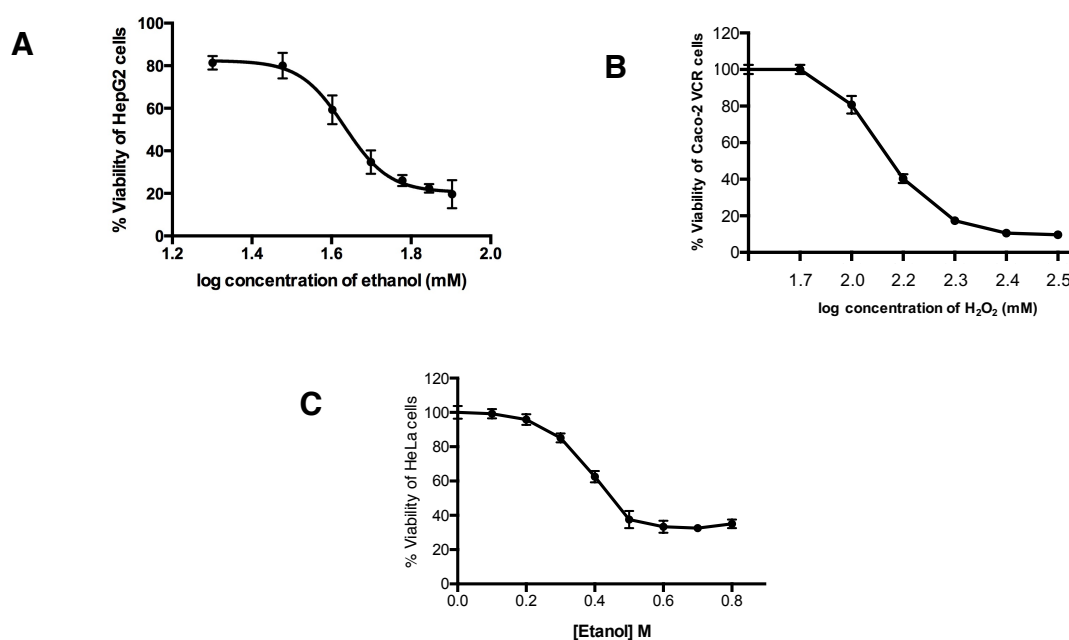


Figure 3- 4 Viability of (a) HepG2, (b) Caco-2 VCR and (c) HeLa cells in presence of different concentrations of ethanol and H₂O₂ (Results show mean ± SEM, N≥3).

Table 3- 3 Tabular summary of cytotoxicity of plant extracts in HepG2, Caco-2 VCR and HeLa cells.

Plant name	Sample ID	% Viability HepG2 (100µg/mL)	% Viability Caco-2 VCR (100µg/mL)	% Viability HeLa (100µg/mL)
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	87.59 ± 0.213	80.96 ± 0.033	-
<i>Artemisia absinthium</i> L.	AA1	81.06 ± 0.099	93.01 ± 0.188	-
<i>Bidens pilosa</i> L.	BP1	92.38 ± 0.021	92.15 ± 0.056	-
<i>Casearia silvestris</i> Sw.	CAS	99.82 ± 0.012	90.36 ± 0.134	-
<i>Cordia verbenaceae</i> A. DC.	CV1	98.16 ± 0.131	99.96 ± 0.091	97.44 ± 0.118
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	99.07 ± 0.010	95.98 ± 0.064	-
<i>Equisetum arvense</i> L.	EA1	87.04 ± 0.055	92.08 ± 0.138	-
<i>Erythrina mulungu</i> Benth.	EM1	95.57 ± 0.101	97.61 ± 0.172	98.13 ± 0.196
<i>Lamium album</i> L.	LA1	96.80 ± 0.053	92.18 ± 0.156	-
<i>Lippia sidoides</i> Cham.	LS1	96.79 ± 0.014	96.85 ± 0.051	95.66 ± 0.235
<i>Malva sylvestris</i> L.	MSF	88.07 ± 0.084	98.93 ± 0.203	-
	MSH	92.88 ± 0.070	94.79 ± 0.202	-
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MI1	97.16 ± 0.108	97.17 ± 0.230	-
<i>Morus nigra</i> L.	MN1	99.81 ± 0.012	97.23 ± 0.129	-
<i>Passiflora incarnata</i> L.	PI1	99.97 ± 0.072	94.69 ± 0.175	-
<i>Persea americana</i> Mill.	PA1	97.12 ± 0.013	99.13 ± 0.113	-
<i>Rhamnus purshiana</i> DC.	RP1	98.34 ± 0.012	89.97 ± 0.320	-
<i>Salix alba</i> L.	SA1	81.68 ± 0.014	83.72 ± 0.074	-
<i>Schinus terebinthifolia</i> Raddi	ST1	84.58 ± 0.045	84.83 ± 0.109	-
<i>Solanum paniculatum</i> L.	SP1	92.47 ± 0.121	98.78 ± 0.080	96.12 ± 0.109
<i>Solidago microglossa</i> DC.	SM1	97.52 ± 0.192	93.49 ± 0.215	-
<i>Syzygium jambolanum</i> (Lam.) DC.	SJ1	93.75 ± 0.048	82.82 ± 0.140	-
<i>Tabebuia avellanedae</i> Lorentz ex Griseb.	TA1	99.74 ± 0.014	82.72 ± 0.111	-
<i>Vernonia polyanthes</i> Less.	VP1	93.04 ± 0.021	95.95 ± 0.079	-

3.4 CYP3A4 MRNA GENE EXPRESSION

3.4.1 Integrity and NanoDrop reading-assessment of the purity and quality of RNA

The two rRNAs 28s and 18s were visible in all RNA samples, which attest its full integrity. Purity and quality of RNA were assessed by differential UV readings as described in section 2.4.3.2.3. The materials were of acceptable quality (ratio ± 2.0) to undergo mRNA amplification as seen in table 3-4.

Samples	Sample ID	RNA concentration (ng/uL)	A260	A280	A260/A280	A260/A230
Control (DMSO 0.2%)	CTR	639.00	18.475	9.237	2.00	2.21
RIF 50 μ M	RIF	657.4	16.434	8.122	2.02	2.07
DMSO 1%	DMS	544.0	13.600	6.754	2.01	2.19
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	269.5	6.738	3.547	1.90	2.31
<i>Artemisia absinthium</i> L.	AA1	645.9	16.147	8.745	1.85	2.31
<i>Bidens pilosa</i> L.	BP1	771.4	19.285	9.628	2.00	2.21
<i>Casearia silvestris</i> Sw.	CAS	570.9	14.272	7.458	1.91	1.87
<i>Cordia verbenaceae</i> A. DC.	CV1	688.4	17.211	8.752	1.97	2.40
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	694.6	17.364	9.028	1.92	2.23
<i>Equisetum arvense</i> L.	EA1	628.2	15.705	8.090	1.94	2.30
<i>Erythrina mulungu</i> Benth.	EM1	573.5	14.337	7.382	1.94	2.43
<i>Lamium album</i> L.	LA1	588.9	14.722	7.382	1.99	2.28
<i>Lippia sidoides</i> Cham.	LS1	691.4	17.284	8.792	1.97	2.19
<i>Malva sylvestris</i> L.	MSF	567.4	14.184	7.210	1.97	2.30
	MSH	628.8	15.721	8.200	1.92	2.29
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MI1	597.1	14.926	7.861	1.90	2.28
<i>Morus nigra</i> L.	MN1	452.8	11.321	5.913	1.91	2.19
<i>Passiflora incarnata</i> L.	PI1	747.9	18.696	10.089	1.86	2.30
<i>Persea americana</i> Mill.	PA1	568.7	14.217	7.206	1.97	2.38
<i>Rhamnus purshiana</i> DC.	RP1	638.3	15.957	8.066	1.98	2.05
<i>Salix alba</i> L.	SA1	461.5	11.537	5.908	1.95	2.08
<i>Schinus terebinthifolia</i> Raddi	ST1	425.8	10.645	5.577	1.91	1.85
<i>Solanum paniculatum</i> L.	SP1	690.3	17.257	8.928	1.93	2.33
<i>Solidago microglossa</i> DC.	SM1	599.1	14.978	7.974	1.88	2.23
<i>Syzygium jambolanum</i> (Lam.) DC.	SJ1	368.1	9.203	4.709	1.95	1.91
<i>Tabebuia avellaneda</i> Lorentz ex Griseb.	TA1	774.6	19.364	9.730	1.99	2.31
<i>Vernonia polyanthes</i> Less.	VP1	654.5	16.363	8.418	1.94	2.40

Table 3- 4 Tabular summary of the purity and quality of RNA using NanoDrop. The ratio of absorbance 260nm and 280nm is used to assess the purity of RNA. The ratio ± 2.0 is generally accepted as "pure" for RNA. The ration 260/230 is used as a secondary mesure of nucleic acid purity and the ratio should also be ± 2.0 .

3.4.2 Real-time qPCR efficiency

Both CYP3A4 and β -actin primers sequences revealed the specificity of the target amplification. The baseline and threshold were properly set. Standard curve demonstrated good regression coefficient and efficiency. Melting curve analysis revealed a single peak for each pair of primers (Figures 3-5, 3-6, 3-7, and 3-8). These results allowed us to select β -actin as potential normaliser gene.

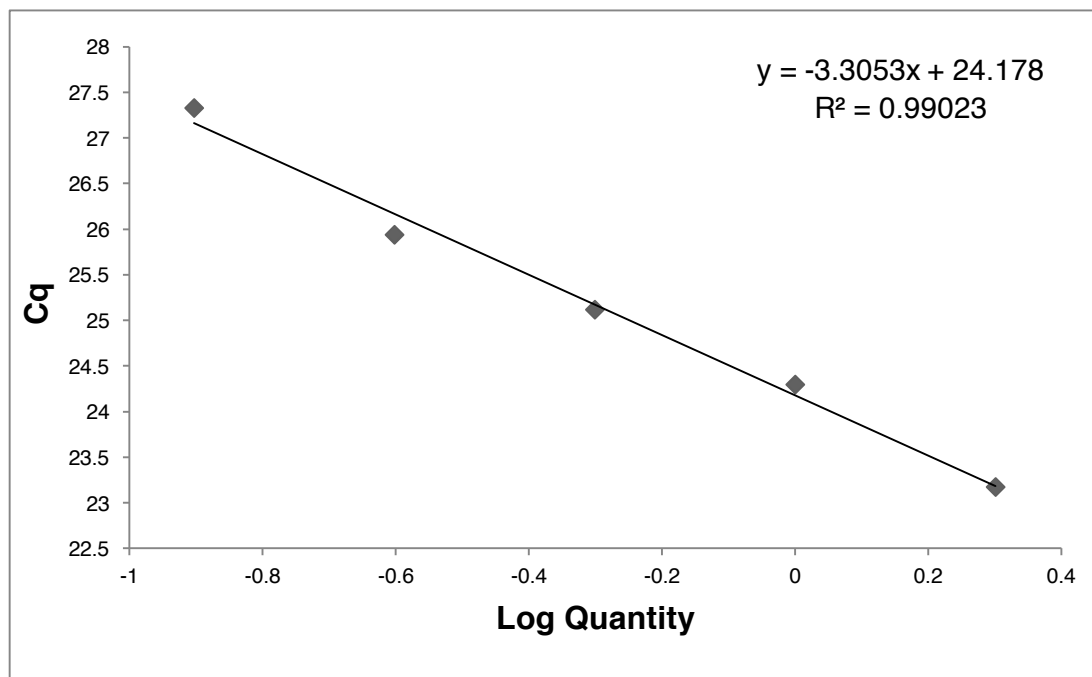


Figure 3- 5 CYP3A4 primer calibration curve.

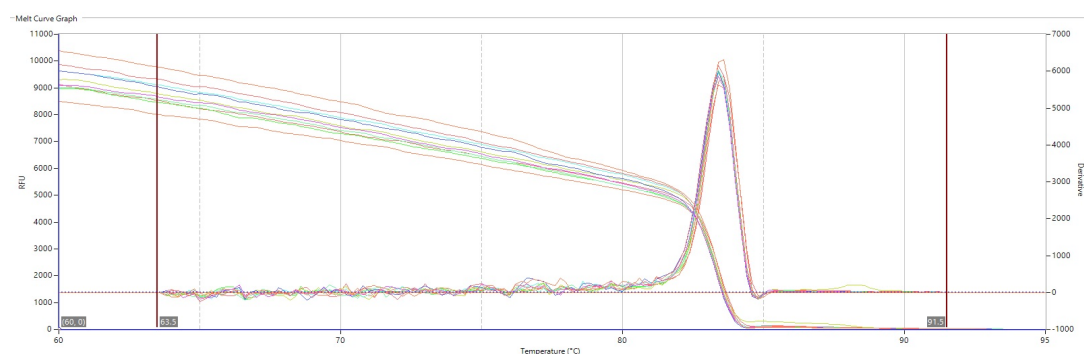


Figure 3- 6 CYP3A4 primer melting curve.

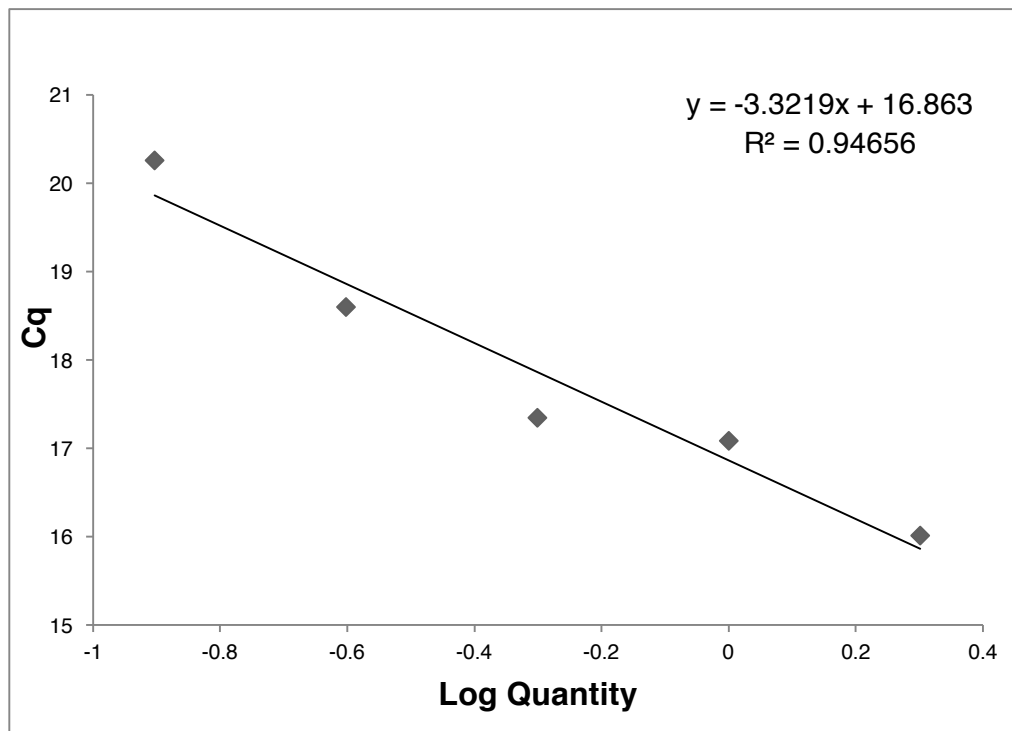


Figure 3- 8 β-actin primer calibration curve.

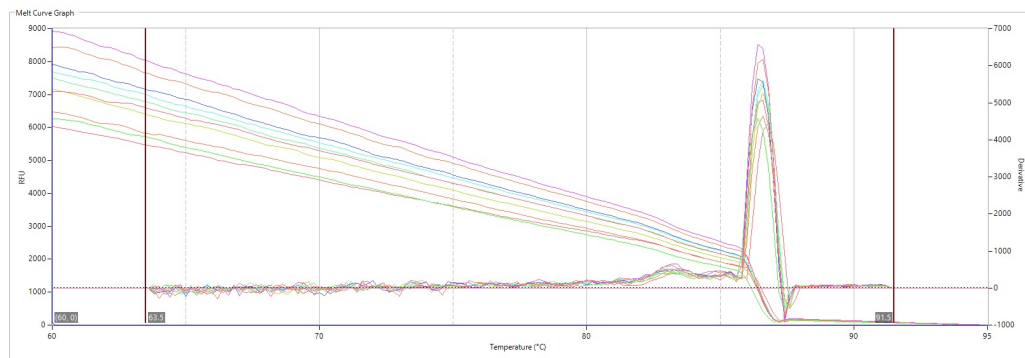


Figure 3- 7 β-actin primer melting curve.

3.4.3 Polymerase chain reaction (PCR)

RIF (50 μ M), a known CYP3A4 inducer, was able to significantly increase CYP3A4 expression in HepG2 cells (4.95-fold; $p < 0.001$). DMSO 1% inhibited CYP3A4 expression in 2.7-fold ($p < 0.01$). Therefore, we used these two drugs as inducer/positive and negative reference drug, respectively, in our studies.

Apuleia ferrea (Mart.) Baill., *Casearia silvestris* Sw., *Equisetum arvense* L., *Maytenus ilicifolia* Mart. ex Reissek, *Passiflora incarnata* L., *Persea americana* Mill., *Rhamnus purshiana* DC., *Salix alba* L., *Schinus terebinthifolia* Raddi, *Syzygium jambolanum* (Lam.) DC. and *Vernonia polyanthes* Less. were able to induce CYP3A4 mRNA expression at significant levels ranging from 1.53 fold (*Rhamnus purshiana* DC.) to 4.88-fold (*Schinus terebinthifolia* Raddi).

Lamium album L. and *Solanum paniculatum* L., on the other hand, inhibited CYP3A4 mRNA gene expression in 1.96 and 2.40-fold respectively, showing a similar effect to DMSO 1% ($p < 0.01$).

Artemisia absinthium L., *Bidens pilosa* L., *Cordia verbenaceae* A. DC., *Costus spicatus* (Jacq.) Sw., *Erythrina mulungu* Benth., *Lippia sidoides* Cham., *Malva sylvestris* L. (flower and herb), *Morus nigra* L., *Solidago microglossa* DC. and *Tabebuia avellanedae* Lorentz ex Griseb. were not able to modulate CYP3A4 expression in a significant manner compared to non-treated cells (Figure 3-9 and Appendix 7.6).

By mimicking the traditional use of the selected herbs, we surprisingly observed that about 54% of the extracts were able to interfere with CYP3A4 mRNA gene expression at the tested concentration of 100 μ g/mL. Out of the extracts that were able to modulate CYP3A4 expression, two are native to Brazil: *Vernonia polyanthes* Less. (induces CYP3A4 expression) and *Solanum paniculatum* L. (inhibits CYP3A4 expression).

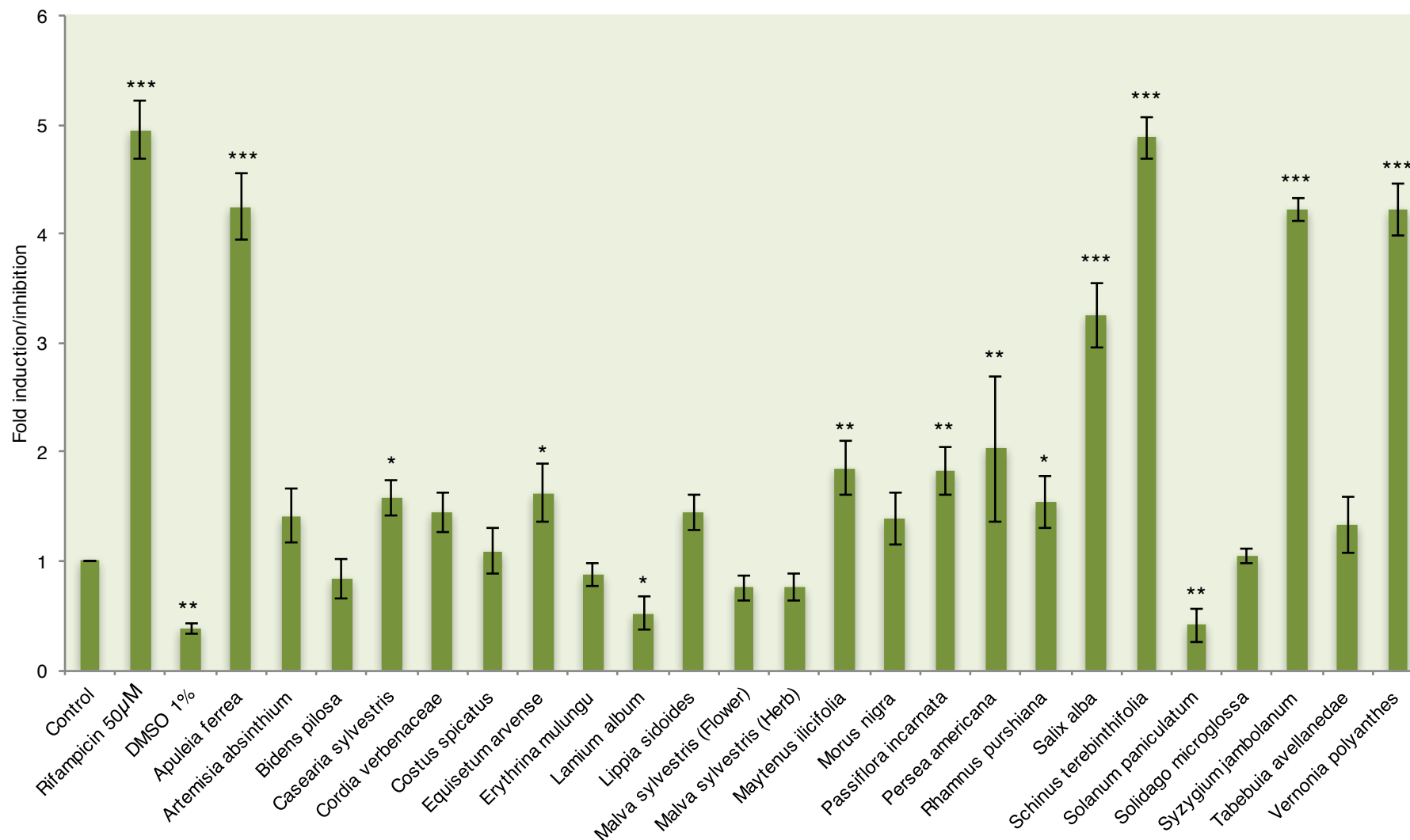


Figure 3- 9 Effect of 24-hour incubation of extracts (100µg/mL) or the positive control rifampicin (50µM) and the negative control DMSO 1% in CYP3A4 mRNA gene expression in HepG2 cells. Thirteen extracts – CAS, EA1, RP1 ($p<0.05$), MI1, PI1, PA1 ($p<0.01$), AF1, SA1, ST1, SJ1 and VP1 ($p<0.001$) produced statistically significant increase in CYP3A4 mRNA gene expression in HepG2 cells based on one-way ANOVA test followed by Bonferroni correction. LA1 ($p<0.05$) and SP1 (0.01), on the other hand, were able to decrease CYP3A4 expression.

3.4.4 hPXR antagonistic effect of *Solanum paniculatum* L.

To further investigate whether the diminished expression of CYP3A4 mRNA gene by *Solanum paniculatum* L. treatment was mediated by an antagonist effect upon hPXR, we performed a reporter gene assay. As we can observe, co-transfected HeLa cells treated with RIF (1 μ M) and serial dilutions of *Solanum paniculatum* L. extract show a dose-response inhibition upon hPXR transcription activity. The maximal inhibition was close to 60% with an IC₅₀ of 0.38mg/mL (Figure 3-10).

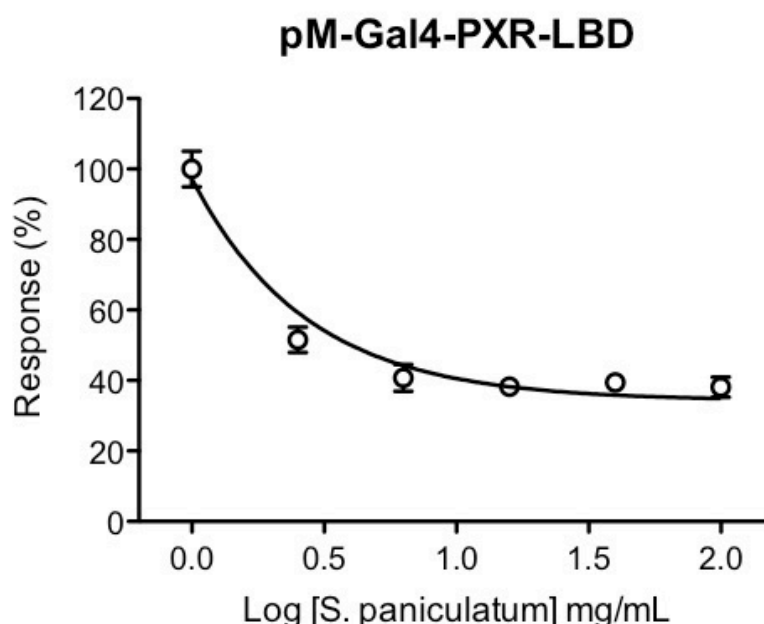


Figure 3- 10 Antagonistic effect of *Solanum paniculatum* L. extract on RIF-induced hPXR activation. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with increasing concentrations of *Solanum paniculatum* L. in the presence of RIF 1 μ M (EC₅₀). Luciferase activity was measured after 24h and normalized as a percentage of RIF treated cells.

Inhibition of hPXR reporter gene assay was not due to interference of the extract with the luciferase activity since it did not show any effect on luciferase activity in HeLa cells transfected with CMV-luciferase expression vector. Additionally, this extract showed an agonist effect in HeLa cells co-transfected with thyroid hormone receptor beta 1 ligand binding domain and Gal4 luciferase reporter treated with triiodothyronine (T3). We did not use *Renilla* luciferase assay as an internal control since several authors have

described the limitations of *Renilla* luciferase as an internal control of transcription efficiency (Figures 3-11, 3-12, and 3-13) (Shifera and Hardin, 2010, Ho and Strauss, 2004).

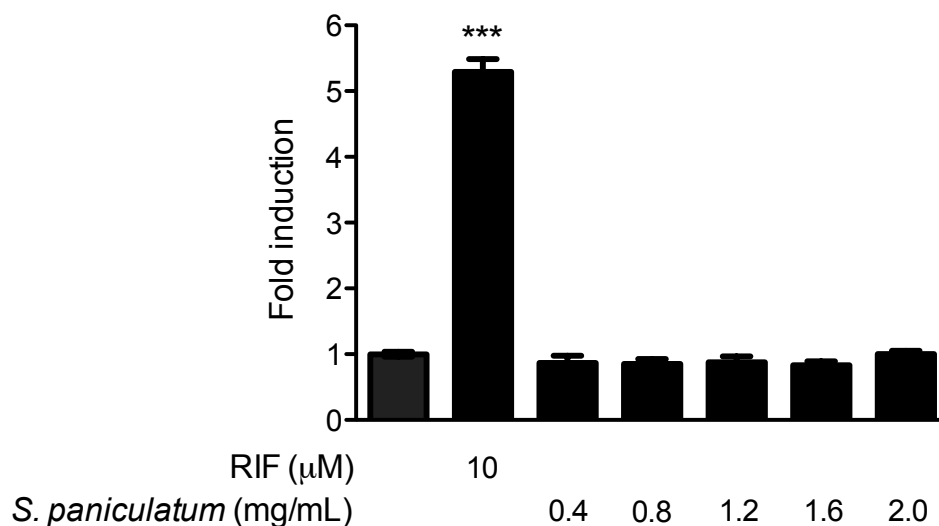


Figure 3- 11 Agonistic effect. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with vehicle, rifampicin (RIF) or increasing concentrations of *Solanum paniculatum* L. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. Results represent data from three independent experiments performed in triplicate. *** $p \leq 0.001$.

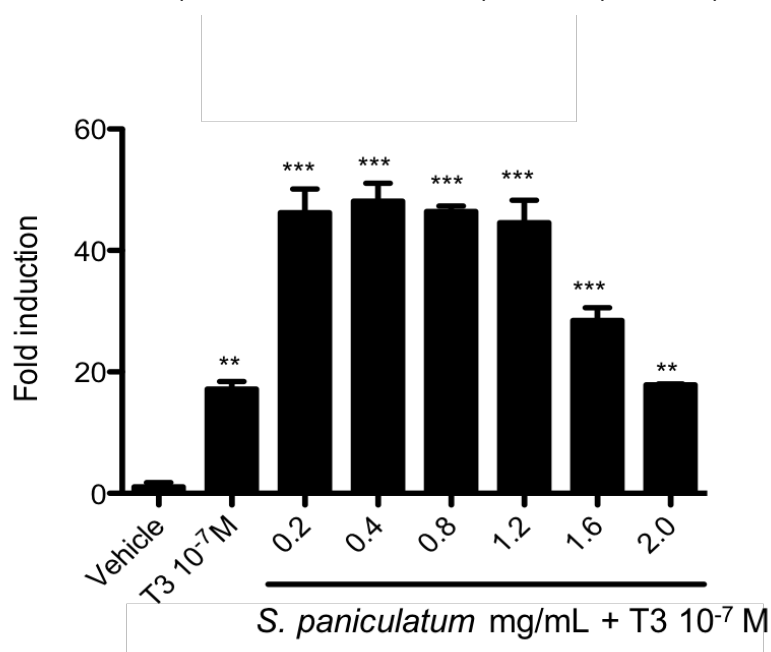


Figure 3- 12 HeLa cells were co-transfected with expression vector pM-Gal4-dTR_{β1}-LBD and Gal4 luciferase reporter and treated with vehicle, T3 or increasing concentrations of *Solanum paniculatum* L. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. ** $p \leq 0.01$; *** $p \leq 0.001$.

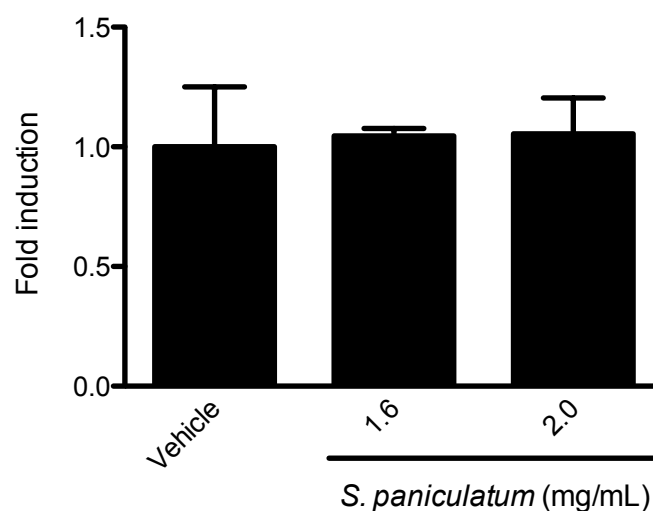


Figure 3- 13 HeLa cells were transfected with expression vector CMV luciferase reporter and treated with vehicle, 1.6 or 2.0 mg/ mL of *Solanum paniculatum* L. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle.

We also evaluated the effect of the other three native extracts (*Erythrina mulungu*, *Lippia sidoides*, and *Cordia verbenaceae*) on hPXR transcription activity. We observed that *Erythrina mulungu* Benth. is an hPXR partial agonist since it increased hPXR reporter gene transcription activation in a dose-dependent manner, but not as strong as RIF. *Lippia sidoides* Cham. did not show any effect on hPXR. *Cordia verbenaceae* A. DC. failed to increase hPXR transcription activity. When we treated the cells with *Cordia verbenaceae* A. DC. extract the transcription activity of RIF was impaired by 50% suggesting an antagonist effect. However, this is due to unspecific effects, since inhibition was also observed with a high affinity thyroid hormone receptor (Figures 3-14 to 3-20).

3.4.6 *Erythrina mulungu* Benth. displays a partial agonistic effect on hPXR luciferase reporter gene assay.

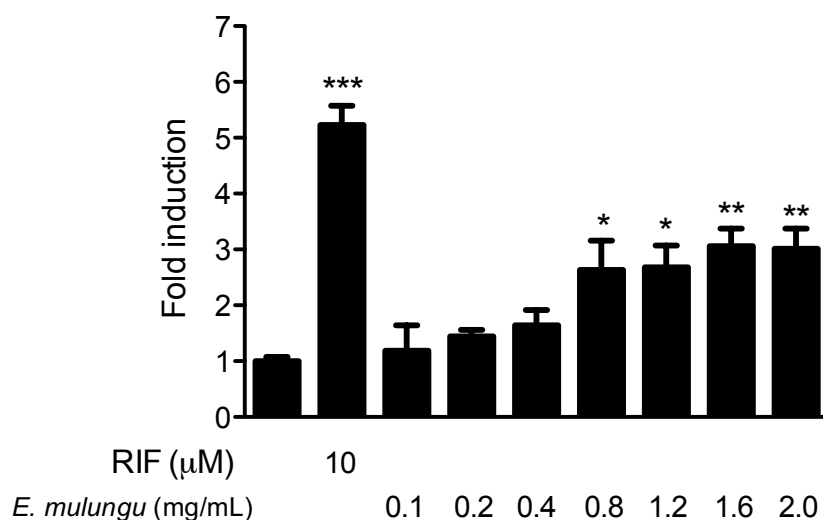


Figure 3- 14 Agonistic effect. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with vehicle, RIF or increasing concentrations of *Erythrina mulungu* Benth. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

3.4.7 *Lippia sidoides* Cham. does not display any effect on hPXR luciferase reporter gene assay.

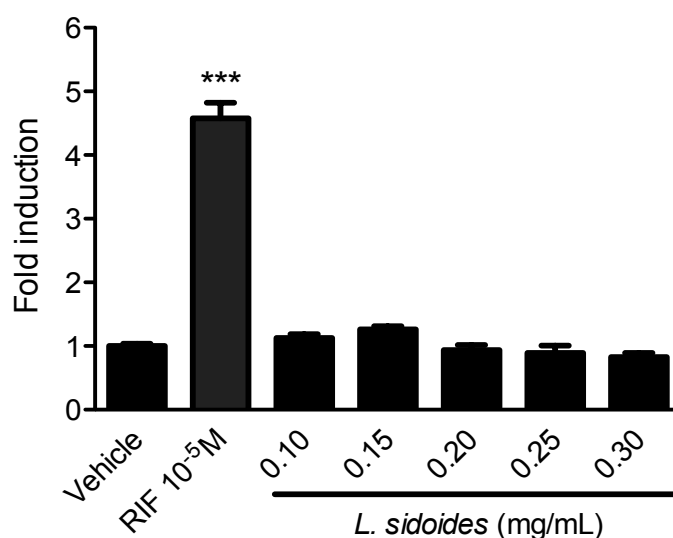


Figure 3- 15 Agonistic effect. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with vehicle, RIF or increasing concentrations of *Lippia sidoides* Cham. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. ***p ≤ 0.001.

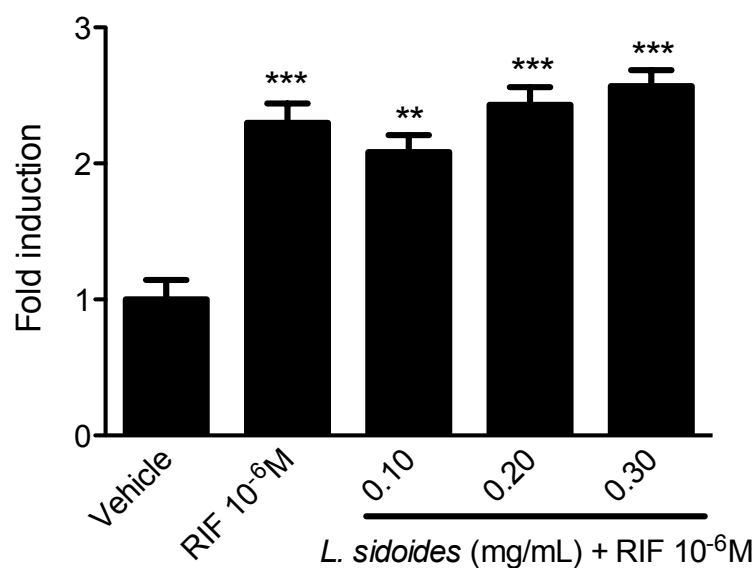


Figure 3- 16 Antagonistic effect. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with vehicle, RIF without and with increasing concentrations of *Lippia sidoides* Cham. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. **p ≤ 0.01. ***p ≤ 0.001.

3.4.8 *Cordia verbenaceae* A. DC. displays an antagonistic effect on both hPXR and TR_{β1} but not CMV luciferase reporter gene assay.

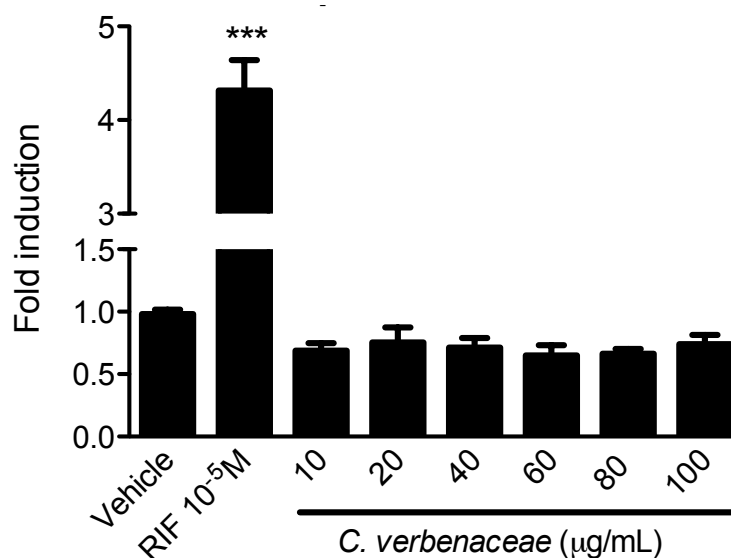


Figure 3- 17 Agonistic effect. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with vehicle, RIF or increasing concentrations of *Cordia verbenaceae* A. DC. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. ***p ≤ 0.001.

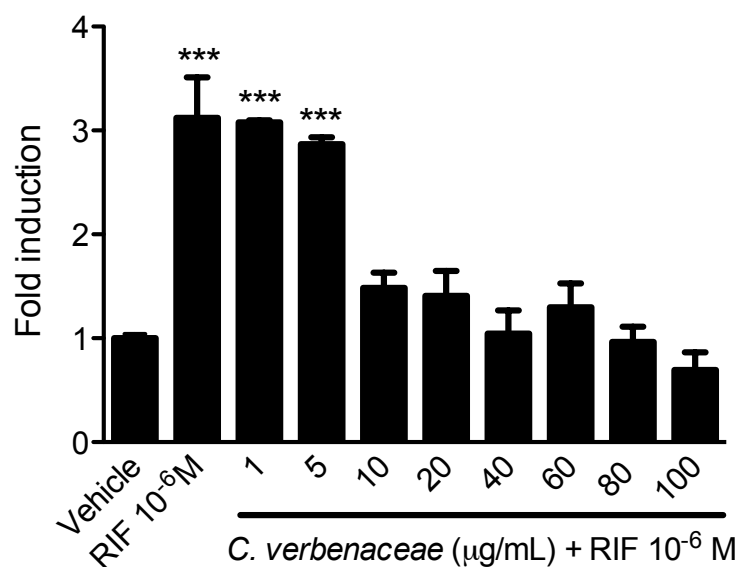


Figure 3- 18 Antagonistic effect. HeLa cells were co-transfected with expression vector pM-Gal4-PXR-LBD and Gal4 luciferase reporter and treated with vehicle, RIF without or with increasing concentrations of *C. verbenaceae*. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. *** $p \leq 0.001$.

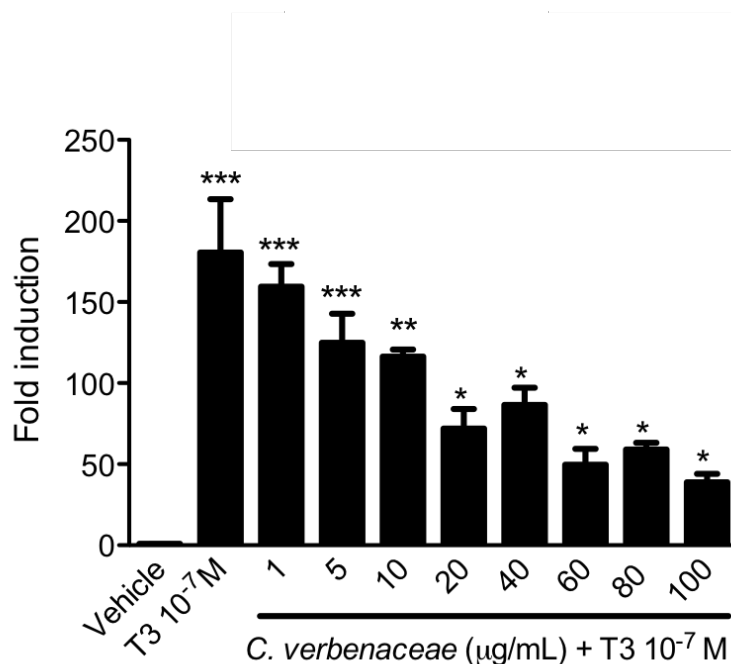


Figure 3- 19 Antagonistic effect on $TR_{\beta 1}$. HeLa cells were co-transfected with expression vector pM-Gal4-d $TR_{\beta 1}$ -LBD and Gal4 luciferase reporter and treated with vehicle, T3 without and with increasing concentrations of *Cordia verbenaceae* A. DC. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

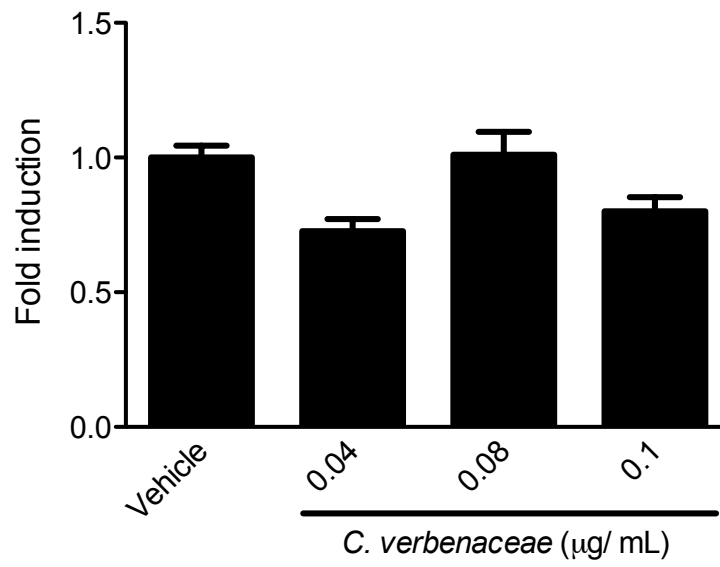


Figure 3- 20 HeLa cells were co-transfected with expression vector CMV luciferase reporter and treated with vehicle or with increasing concentrations of *Cordia verbenaceae* A. DC. Luciferase activity was measured after 24 hours and reported as fold induction compared to vehicle.

3.5 ESTIMATION OF INTRACELLULAR GSH LEVELS IN HEPG2 CELLS

The rate of change in the kinetic absorbance of TNB for the different GSH standard concentrations is linearly proportional to the amount of GSH in each sample. Thus, a graph plot of the slopes of each GSH standard concentration against the GSH concentration, otherwise known as the F-slope curve can be used to estimate the amount of GSH in the treated wells given their individual slopes (Figures 3-21 and 3-22).

The GSH concentration obtained for the control cells was $29.91 \pm 0.36\mu\text{M}$. Twenty extracts- *Apuleia ferrea* (Mart.) Baill., *Bidens pilosa* L., *Casearia silvestris* Sw., *Cordia verbenaceae* A. DC., *Costus spicatus* (Jacq.) Sw., *Equisetum arvense* L., *Lamium album* L., *Lippia sidoides* Cham., *Malva sylvestris* L. (flower and herb), *Maytenus ilicifolia* Mart. Ex Reissek, *Passiflora incarnata* L., *Persea americana* Mill., *Rhamnus purshiana* DC., *Salix alba* L., *Schinus terebinthifolia* Raddi, *Solanum paniculatum* L., *Solidago microglossa* DC., *Tabebuia avellanedae* Lorentz ex Griseb. and *Vernonia polyanthes* Less. - decreased the intracellular GSH concentration beyond levels seen in the control cells. This decrease was found to be statistically significant ($P \leq 0.05$, 0.01 and 0.001). The majority of the extracts decreased GSH levels at a comparable level to that produced by the positive control BSO, which decreased intracellular GSH levels to $17.78 \pm 1.70\mu\text{M}$ ($P \leq 0.001$). Only one extract - *Erythrina mulungu* Benth. - was able to increase GSH levels at a significant level 47.05 ± 2.0 (Figure 3-23 and Appendix 7.7).

Similar to what has been observed on CYP3A4 mRNA gene expression, native Brazilian extracts were also able to cause changes in GSH levels. *Cordia verbenaceae* A. DC., *Lippia sidoides* Cham., and *Solanum paniculatum* L. depleted GSH levels whereas *Erythrina mulungu* Benth. displayed the opposite effect.

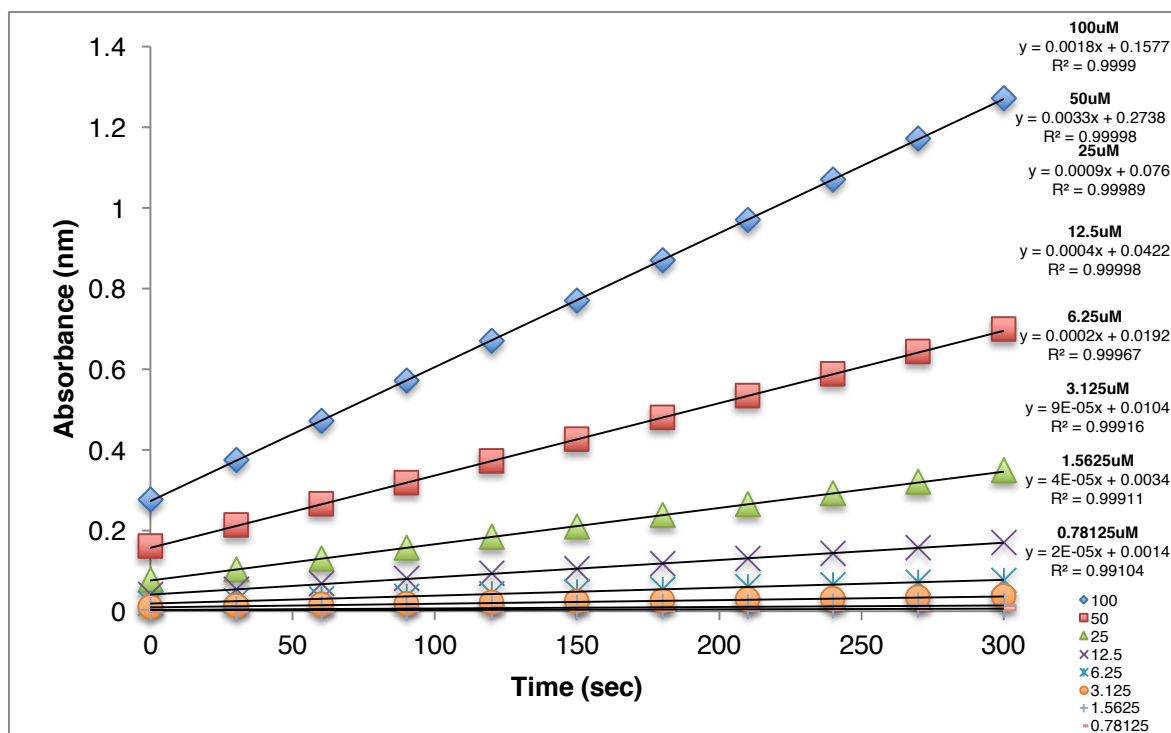


Figure 3- 21 Graph plot of the rate of change in TNB absorbance at the different GSH standard concentrations.

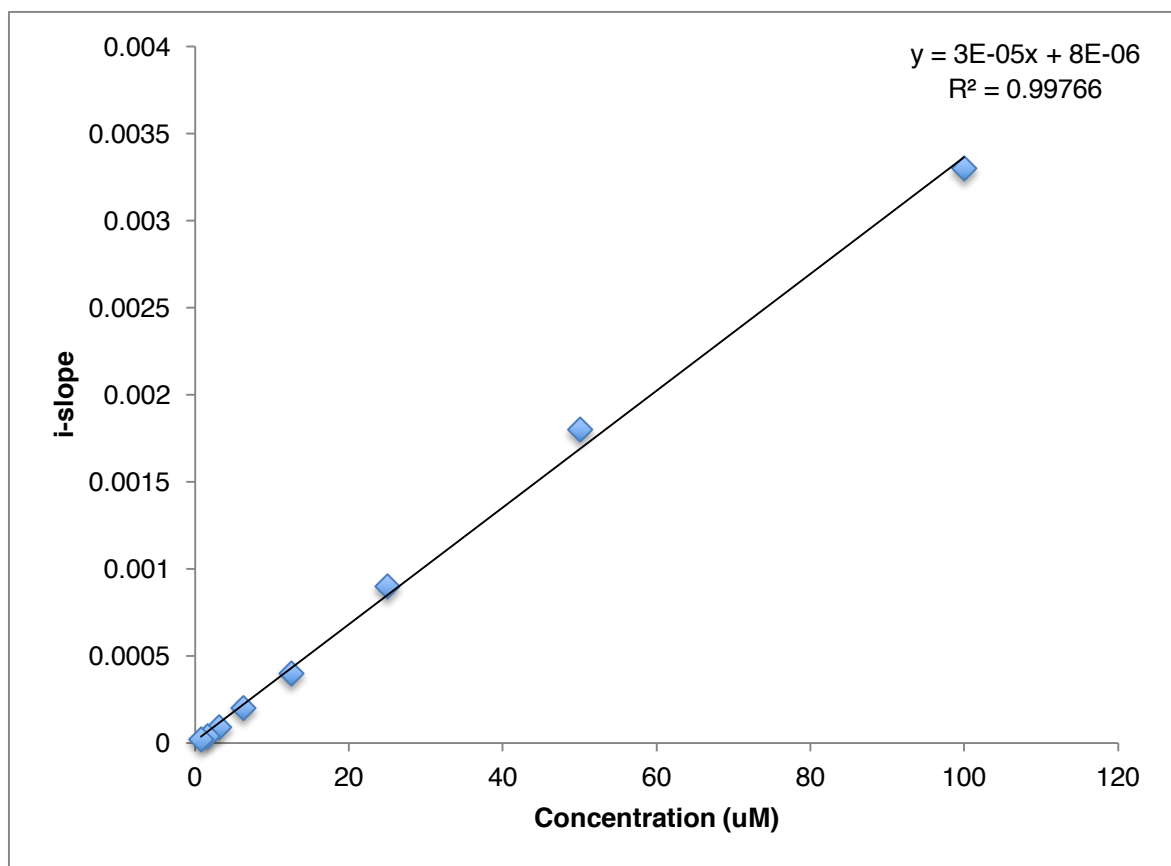


Figure 3- 22 The F-slope is created according to the values of each standard concentration obtained in figure 3- 21, which is used to estimate the amount of GSH in each sample.

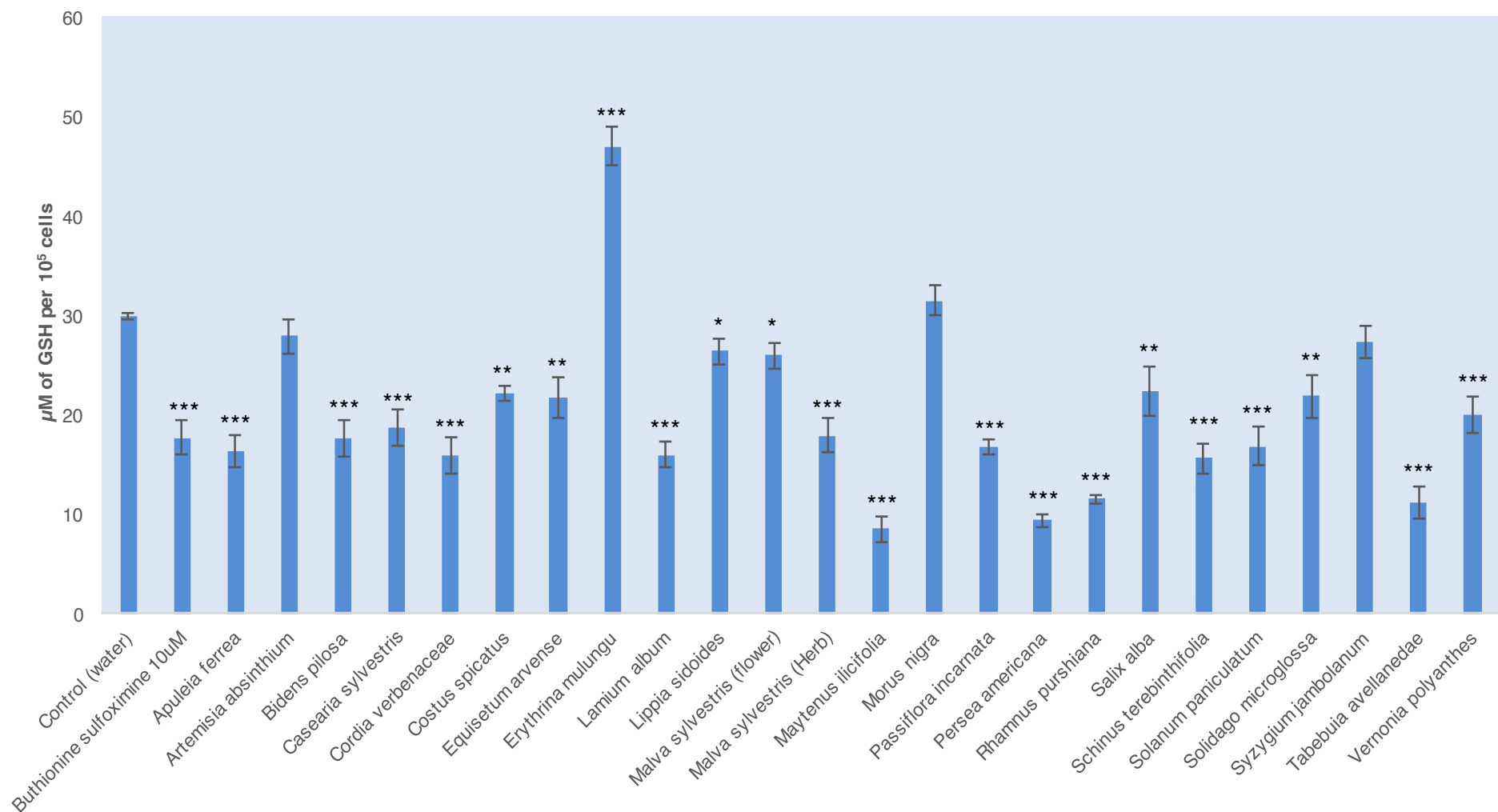


Figure 3- 23 Effect of 24-hour incubation of extracts (100µg/mL) on the intracellular GSH levels in HepG2 cells. Twenty extracts (AF1, BP1, CAS, CV1, CS1, EA1, LA1, LS1, MSF, MSH, MI1, MN1, PI1, PA1, RP1, SA1, ST1, SP1, SM1, SJ1, TA1 and VP1) produced statistically significant ($p \leq 0.05$, 0.01 and 0.001) decrease in GSH concentrations based on one-way ANOVA test followed by Bonferroni correction. Only one extract EM1 was able to increase intracellular GSH levels.

3.6 GGT ACTIVITY

The GGT activity in HepG2 cells was measured according to the amount of *p*-nitroaniline liberated in the cell surface transferase reaction and calculated using a calibration curve.

The GGT inhibitor acivicin (5 μ M) was able to significantly decrease GGT activity by almost 70% ($p < 0.001$) compared to non-treated cells. *Cordia verbenacea* A. DC., *Persea americana* Mill., *Salix alba* L. and *Syzygium jambolanum* (Lam.) DC. lowered GGT activity by 48%, 31%, 36% and 35% respectively ($p < 0.01$). *Costus spicatus* (Jacq.) Sw. and *Schinus terebinthifolia* Raddi were also able to inhibit GGT activity by 33% and 32% respectively ($p < 0.05$). Decreased GGT activity by these extracts except for *Syzygium jambolanum* (Lam.) DC. indicates a possible correlation with this effect and the observed depletion of GSH levels.

On the other hand, *Apuleia ferrea* (Mart.) Baill., *Passiflora incarnata* L. and *Rhamnus purshiana* DC. significantly increased GGT activity in 63% ($p < 0.001$), 33% ($p < 0.05$) and 60% ($p < 0.001$) respectively (Figure 3-24 and Appendix 7.8). Although the GGT activity in the cells treated with these plant extracts was increased, GSH levels were also lowered by the same samples. Therefore, this depletion may be correlated to another mechanism.

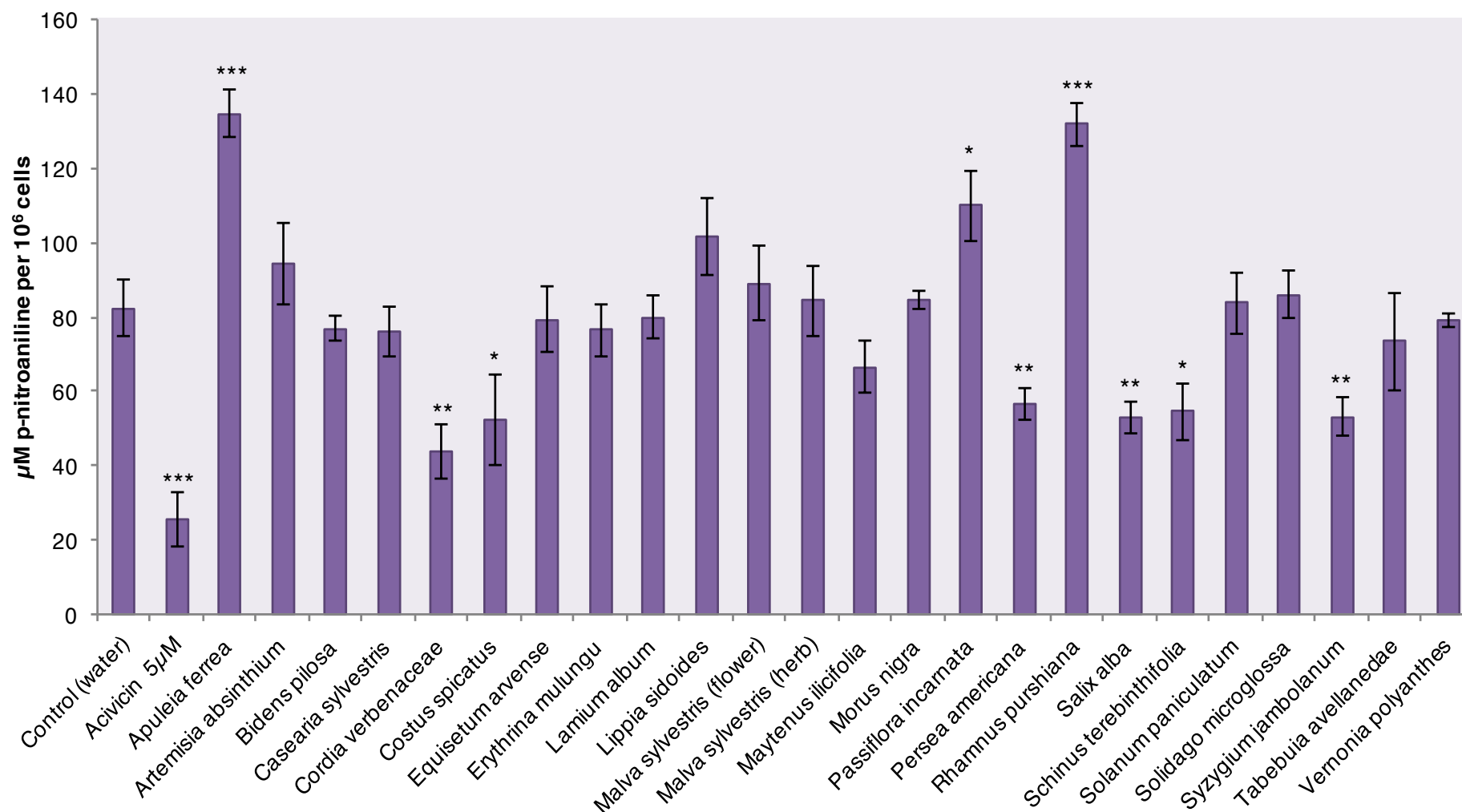


Figure 3- 24 Effect of 24-hour incubation of extracts (100µg/mL) on the GGT activity in HepG2 cells. Six extracts – CS1, ST1 ($p < 0.05$), CV1, PA1, SA1, SJ1 ($p < 0.001$) produced statistically significant decrease in GGT activity based on one-way ANOVA test followed by Bonferroni correction. PI1 ($p < 0.05$), AF1 and RP1 ($p < 0.001$), on the other hand, were able to increase GGT activity.

3.7 MODULATION OF P-GLYCOPROTEIN DEPENDENT RH-123 EFFLUX IN CACO-2 VCR CELLS

The intracellular Rh-123 fluorescence in the cells incubated with *Apuleia ferrea* (Mart.) Baill., *Salix alba* L., *Schinus terebinthifolia* Raddi and *Syzygium jambolanum* (Lam.) DC. was significantly higher than cells treated only with Rh-123. This indicates increased efflux inhibition by these extracts.

The effect of *Salix alba* L. (192.5%) and *Syzygium jambolanum* (Lam.) DC. (182.39%) in the efflux activity was relatively similar to the one found in the positive control verapamil (181.78%). The Rh-123 fluorescence in Caco-2 VCR cells was higher in *Apuleia ferrea* (Mart.) Baill. (412.72%) and *Schinus terebinthifolia* Raddi (442.61%), indicating strong efflux inhibition caused by these extracts (Figure 3-25 and Appendix 7.9).

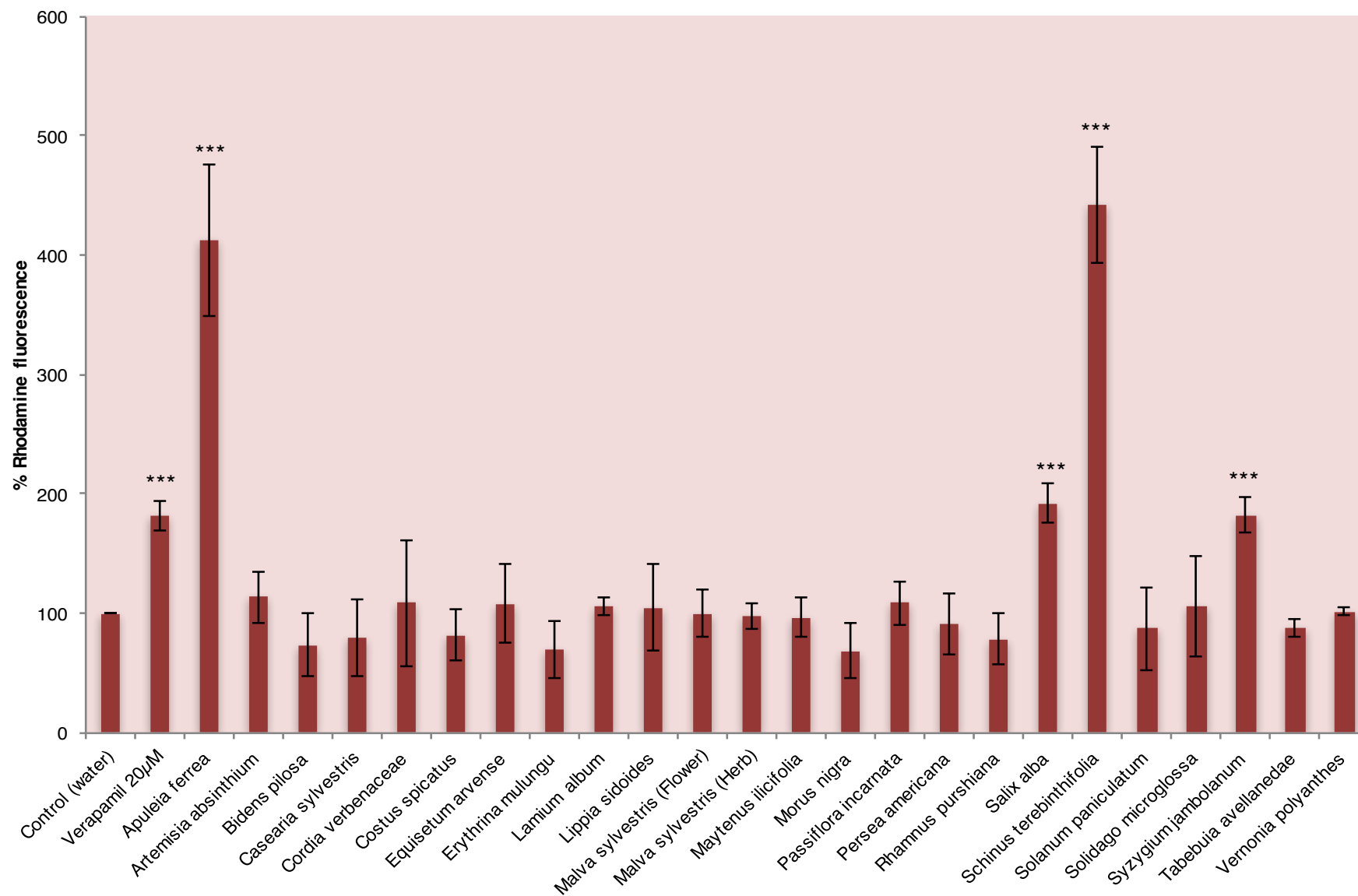


Figure 3- 25 Increased intracellular Rh-123 concentration in Caco-2 VCR cells in the presence of either extracts or verapamil (20µM). Four extracts – AF1, SA1, ST1 and SJ1 – were able to significantly increase Rh-123 concentration in the cells ($p < 0.001$). One-way ANOVA followed by Bonferroni's post test was carried out to determine the significance of extracts.

3.8 SUMMARY OF THE EFFECTS OF SELECTED RENISUS MEDICINAL PLANTS IN CYP3A4, HPXR, GSH, GGT AND P-GP

The effects of the twenty-four plant extracts assessed in our study in the selected targets have been displayed in this section. In order to provide a clear overview of our work, we summarise our results in table 3-5: it outlines the effects (many multiple, some absent) of the researched extracts on the researched targets.

Table 3- 5 Effects of RENISUS plant species in the *in vitro* studied targets.

<i>RENISUS plant species</i>	<i>Sample ID</i>	<i>Type(s) of interaction(s)</i>
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	↑CYP3A4, ↓GSH, ↑GGT, ↓P-gp efflux
<i>Artemisia absinthium</i> L.	AA1	No effects on the researched targets
<i>Bidens pilosa</i> L.	BP1	↓GSH
<i>Casearia silvestris</i> Sw.	CAS	↑CYP3A4, ↓GSH
<i>Cordia verbenaceae</i> A. DC.	CV1	↓GSH, ↓GGT, *
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	↓GSH, ↓GGT
<i>Equisetum arvense</i> L.	EA1	↑CYP3A4, ↓GSH
<i>Erythrina mulungu</i> Benth.	EM1	↑GSH. *
<i>Lamium album</i> L.	LA1	↓CYP3A4, ↓GSH
<i>Lippia sidoides</i> Cham.	LS1	↓GSH, *
<i>Malva sylvestris</i> L.	MSF	↓GSH
	MSH	↓GSH
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MI1	↑CYP3A4, ↓GSH
<i>Morus nigra</i> L.	MN1	No effects on the researched targets
<i>Passiflora incarnata</i> L.	PI1	↑CYP3A4, ↓GSH, ↑GGT
<i>Persea americana</i> Mill.	PA1	↑CYP3A4, ↓GSH, ↓GGT
<i>Rhamnus purshiana</i> DC.	RP1	↑CYP3A4, ↓GSH, ↑GGT, ↓P-gp efflux
<i>Salix alba</i> L.	SA1	↑CYP3A4, ↓GSH, ↓GGT
<i>Schinus terebinthifolia</i> Raddi	ST1	↑CYP3A4, ↓GSH, ↓GGT, ↓P-gp efflux
<i>Solanum paniculatum</i> L.	SP1	↓CYP3A4*, ↓GSH
<i>Solidago microglossa</i> DC.	SM1	↓GSH
<i>Syzygium jambolanum</i> (Lam.) DC.	SJ1	↑CYP3A4, ↓GGT, ↓P-gp efflux
<i>Tabebuia avellanedae</i> Lorentz ex Griseb.	TA1	↓GSH
<i>Vernonia polyanthes</i> Less.	VP1	↑CYP3A4, ↓GSH

* antagonistic effect on hPXR

* partial agonistic effect on hPXR

* no effect on hPXR

4 GENERAL DISCUSSION

4.1 IN VITRO STUDIES OF RENISUS MEDICINAL PLANTS IN THE SELECTED TARGETS

We present for the first time *in vitro* preclinical data for a selection of RENISUS medicinal plants. These plants were assessed for their effects on selected metabolic and transporter targets as a step towards producing their potential HDI profile. While some of these plants are native to Brazil, the majority are consumed all around the world by many people on a daily basis; these results, therefore, not only may help Brazil to better integrate the use of these plants into clinical practice, but will also certainly prompt further research in Europe, North America and worldwide.

It was necessary to balance a number of factors in the experimental design. These factors included the nature of the samples (herbal extracts), their number, as well as a lack of consensus between the academy, regulatory bodies, and industry.

Our results show that 22 out of the 24 plant extracts screened have a significant effect with at least one of the selected targets and therefore have the potential to interact with essential drugs prescribed in Brazil. These metabolic HDI may also be complicated by intrinsic factors such as genetic polymorphisms, disease, age, and sex.

Taking into account all these aspects, we will discuss in the following pages the rationale behind our experimental approach, the interpretation of our results within the context of HDI, and their potential clinical impact in the case of Brazil.

4.1.1 Experimental approaches to the *in vitro* metabolism and transport studies

Drug metabolism studies are an essential step in the preclinical development of medicines. To this end, *in vitro* models must provide vital information on the effects of the lead drug candidate (LDC) upon drug metabolising enzymes, such as the CYP, as a step towards predicting potential DDI. Many *in vitro* experimental models of drug metabolism and disposition make use of cells; HH or their microsomes (HLM), for example, are considered the best models for enzyme inhibition, whereas HLM is the preferred test system for enzyme induction. Caco-2 cells are a common choice for transporter studies (Zhang et al., 2012a). Several other models have been successfully developed to replace the use of cells, and have been validated for predicting the effects of single drug entities in the metabolism with similar correlation to HLM. Such is the case of characterised metabolic recombinant enzymes (CYP1A2, 2C9, 2C19, 2D6, and 3A4) expressed in *Escherichia coli*, which has been developed by the pharmaceutical company AstraZeneca (McGinnity and Riley, 2001). The use of *in silico* models has become more common in the pharmaceutical industry in recent years to speed up the selection of the best lead candidates at this stage of drug development (Steinmetz and Spack, 2009).

In order to be accepted by regulatory bodies, drug metabolism models must be robust, validated and reproducible. However, unlike the requirements for synthetic drug studies, there is no regulatory requirement for preclinical metabolism studies of traditional herbal medicines and no substantial industrial activity exists on this side. Metabolism studies on traditional herbal medicines are normally only initiated upon the receipt of case reports documenting a supposed interaction, which trigger warnings from the regulators. Otherwise, such studies originate from purely academic-driven programmes focused on demonstrating the potential effects of traditional herbal medicines on metabolic and transporter targets (Brantley et al., 2014); these studies are generally carried out by adapting protocols to their own needs, thus leading to an array of different experimental approaches.

The established principles and models used for metabolism studies in synthetic drugs are directly applicable to herbal medicines (He et al., 2010). However, they would require significantly high budgets and infrastructure. Due to the particular scope of the academic activities, a case can thus be made regarding the choice of cell lines. HH are generally replaced by other cell models like HepG2 and HepaRG cells for determination of metabolic activities. The hepatoma cell line HepG2 is one of the most popular models for pharmacological and toxicological *in vitro* studies in academia despite having lost some liver functions, and its supply is readily available from any public cell bank (Guo et al., 2011). The HepaRG cell line is derived from another hepatocellular carcinoma cell and was selected for our project because it maintains important metabolic activities lost in HepG2 cells. But it is significantly more expensive and only available through a private company and still has lost many functions when compared to HH (Szabo et al., 2013).

The high costs of HepaRG and HH cells forces many researchers to use the cheaper HepG2 cell line. A vial containing $2-3 \times 10^6$ HepG2 cells costs around 330 GBP and stocks can be made for further use whenever they are needed. On the other hand, a vial containing 10 million HepaRG cells would cost nearly double at about 600 GBP, and cells would have to be plated out in 24-well plates for immediate use. Furthermore, HepG2 is an immortal cell line and therefore it is suitable for long-term maintenance. It generally reaches 70 – 80% confluence within 48 hours, which allows the conduction of medium and high throughput screenings. Long-term maintenance of HepaRG and HH with high availability, on the other hand, could be very challenging in traditional two-dimensional monolayer cultivation systems (Klein et al., 2014).

Caco-2 cells are one of the most used *in vitro* models for the assessment of P-gp transporter activity within most pharmaceutical industries (Eneroth et al., 2001) and reports of use of Caco-2 for transporter studies in herbal medicines have been also well documented (Li et al., 2014, Wu et al., 2016, Yoshida et al., 2006). The reasons for choosing this cell model for efflux studies are similar to those that were applied to HepG2: the reduced costs and rapid availability for immediate use.

The budget available for preclinical studies and the number of samples to be analysed, therefore, limit the approaches that researchers may select in terms of cell models and methodologies applied to them. In our case, the annual budget of 5,000 GBP per year (3 years) restricted us to the screening of a maximum of twenty-four plant extracts in six different cell-based assays using both HepG2 and Caco-2 cells. Samples in triplicates and quadruplicates had to be run at least three times to optimise experiments. With the exception of real-time polymerase chain reaction (RT-PCR), our experiments were carried out using more affordable colorimetric and fluorimetric assays in 96-well plates in order to maximise the number of samples analysed per time.

GSH is unique among the phase II conjugation mechanisms due to its role in the conjugation of phase I metabolites and in the protection of the body against several diseases. This was determinant for the selection of this target in our studies. P-gp was chosen due to its well-known efflux activity that can affect the absorption of many drugs; indeed, this is an important studied target in the industry during drug discovering stage.

Conduction of CYP3A4 gene expression was made using RT-PCR, which is the most used technique for such a study. Due to the costs of RT-PCR reagents, it was necessary to focus our phase I metabolism study on the expression of CYP3A4 despite other CYPs because it is responsible for metabolising more than 50% of the currently marketed drugs. The fundamentals of RT-PCR have been previously covered in section 2.4.3. Despite the availability of several protocols for HH, the numerous steps and reagents used to produce the cDNA and to amplify the CYP3A4 gene had to be all optimised as this technique is highly dependent on the instrumentation and the cell line.

The studies found in the literature made use of different concentrations of the gene inducer, total RNA and time of the treatment to achieve significant levels of CYP3A4 expression with different cell lines. For instance, Li and coworkers (2013) used RT-PCR for determination of the CYP expression in HepG2 cells by using 1 μ g of total RNA. The amount of RIF used to achieve 2.5 fold induction was 10 μ M for 48 hours treatment (Li et al., 2013). Modarai and

coworkers (2011), on the other hand, needed to use 2.5 μ g of total RNA and RIF 50 μ M treatment over a period of 96 hours of exposition to achieve 3.83-fold induction also in HepG2 cells (Modarai et al., 2011a). Lau and coworkers (2013) conducted a research on the effects of ethanol extracts of the Chinese herbs *Oldenlandia diffusa* (Willd.) Roxb. (Rubiaceae), *Codonopsis tangshen* Oliv. (Campanulaceae), *Rehmannia glutinosa* (Gaertn.) DC. (Plantaginaceae) and *Astragalus propinquus* Schischkin (Leguminosae) on CYP3A4 expression using human colon adenocarcinoma-derived LS180 cells. In that experiment, the amount of RNA used for cDNA synthesis was not disclosed but a 24 hours treatment with RIF 10 μ M was reportedly enough to achieve 8-fold induction (Lau et al., 2013). Likewise, Liu and coworkers (2012) did not indicate the amount of total RNA used but their treatment of the colon adenocarcinoma LS174T cells with RIF 20 μ M for 24 hours resulted in 3-fold induction (Liu et al., 2012).

Contrary to the study conducted by Li and coworkers (2013), the treatment of our HepG2 cells using RIF 10 μ M for 48 hours did not significantly change the CYP3A4 expression using 1 μ g of total RNA. In order to increase CYP3A4 expression using this inducer, some researchers may decide to increase the amount of total RNA amplified, as was the case in Modarai and coworkers (2011). However, we managed to achieve almost 5-fold induction by treating our HepG2 cells using the same RIF concentration for the same time treatment as Modarai and coworkers (2011) and the same total RNA concentration used by Li and coworkers (2013).

The achievement of the optimum protocol for CYP3A4 expression using HepG2 allowed the assessment of the twenty-four plant extracts. *Solanum paniculatum* L. and *Lamium album* L. were the only herbal extracts that decreased CYP3A4 gene expression. In section 3.4.4 we demonstrated that this down-regulation in *Solanum paniculatum* L. is due - at least in part - to an antagonistic effect of the plant extract on hPXR (Mazzari et al., 2016).

Although the method proved to be robust and reproducible, our final results must be put into the context of the limitations of HepG2 as a model for induction studies (Gerets et al., 2012): the poor response to induction was

overcome by the sensitivity of RT-PCR and to the adjustment of the concentration of the controls (Mazzari et al., 2016).

Herbal medicines, like any other xenobiotic, are also able to act as agonists or antagonists of hPXR and consequently modulate the expression of drug metabolising enzymes and transporters. A study published by Awortwe and coworkers (2015) demonstrated that the crude extract and fractions of *Echinacea purpurea* (L.) Moench (Compositae) up-regulated CYP1A2, CYP3A4 and MDR1 gene expression by activation of hPXR in HepG2 cells (Awortwe et al., 2015). Yeung and coworkers (2008) also demonstrated that *Ginkgo biloba* extract was able to induce CYP3A4, CYP3A5 and ABCB1 gene expression by activating the hPXR in LS180 cells (Yeung et al., 2008).

As previously mentioned in sections 1.4.1 and 2.4.4.1, the nuclear receptor hPXR is responsible for the transcription of numerous metabolising enzymes. However, in some cases, the transcription of enzymes may be initiated via the activation of another receptor. For example, a study conducted by Westerink and Schoonen (2007) found that the level of expression of CYP3A4 increased after the treatment of HH and HepG2 cells with hPXR activators but this was not observed for CYP2C8. Using hPXR agonists such as RIF, the expression of CYP3A4 was increased in both HH and HepG2. On the other hand, overexpression of CYP2C8 after hPXR agonist treatment was detectable in HH but not in HepG2 cells (Westerink and Schoonen, 2007). Therefore, expression of CYP2C8 in HepG2 cells might happen via activation of a different receptor, such as CAR.

Several techniques such as flow cytometry and colorimetric assays have been used and published by academic researchers for the measurement of intracellular GSH levels. For example, Staal and coworkers (1992) demonstrated that it is possible to measure the amount of intracellular GSH in T cells in HIV-infected individuals using a flow cytometry protocol (Staal et al., 1992). GSH levels have been also assessed using flow cytometry in cell lines like human acute lymphoblastoid leukaemia cells (Morales et al., 2005), MCF-7 (mammary carcinoma), HCT-8 (colon carcinoma) and MGH-U1 (bladder carcinoma) (Hedley and Chow, 1994). The sensitivity of the technique is the

biggest advantage of flow cytometry. However, the operational costs and the expenses involving the purchasing of materials are the biggest drawbacks.

For the analysis of the effects of twenty-four plant extracts on intracellular GSH levels, it was necessary to find protocols that would allow the assessment of multiple samples at the same time and at a low cost. Among the protocols for determination of intracellular GSH levels using colorimetric assays, we found a suitable kinetic assay published by Allen and coworkers (2000) wherein intracellular GSH levels were analysed in 96-well microtiter plates. This protocol allowed us to work with six different samples at the same time in triplicates. Another similar protocol published by Rahman and coworkers (2007) was used to compare the results found with the Allen and coworkers (2000) protocol. Both procedures were merged into a single method, which was used by our group.

Recycling methods have been previously used by academic researchers to evaluate the effects of herbal medicines on GSH levels. Guan and coworkers (2012) for instance used the recycling assay to assess the effects of liquiritin apioside (LA), the main flavonoid component from *Glycyrrhiza uralensis* Fisch. (*Leguminosae*) on adenocarcinomic human alveolar epithelial cells (Guan et al., 2012). Yoon and coworkers (2012) conducted a study in rat liver homogenates previously treated with the water extract of *Agrimonia eupatoria* L. (*Rosaceae*) by using the recycling method (Yoon et al., 2012). The HepG2 cell line has also been used by Kang and coworkers (2011), who performed the recycling kinetic assay to quantify the amount of GSH in HepG2 cells treated with the wild vegetable *Youngia denticula* Kitam (Kang et al., 2011).

The implementation of the GSH recycling method in our study showed that, with the exception of *Apuleia ferrea* (Mart.) Baill., *Erythrina mulungu* Benth., *Morus nigra* L. and *Syzygium jambolanum* (Lam.) DC., all the remaining tested extracts were able to lower intracellular GSH levels, an important metabolite involved in the clearance of xenobiotics and the detoxification of reactive species (Xie, 2009). The variations of intracellular GSH may be related to different effects:

(a) the inhibition/induction of enzymes involved in its biosynthesis, such as glutamate-cysteine ligase (GCL) and glutathione synthase (Lu, 2013);

(b) the increase (or the inhibition) of GSH efflux from cells (Aw et al., 1986, Lu et al., 1990, Fernandez-Checa et al., 1988) which may also be associated with cell death (De Nicola and Ghibelli, 2014);

(c) the formation (and the possible release) of GSH-adducts which could also cause a reduction of the detectable intracellular GSH (if it is not compensated by a new GSH synthesis) (Blair, 2010);

(d) a modulation of GST activity which could also explain changes in both intra- and extracellular GSH levels (Tolson and Wang, 2010).

Regarding the first possibility (a), the presence of active compounds in the extracts could be depleting GSH in a similar manner BSO does, that is, by inhibiting the GCL enzyme (Marengo et al., 2008). In order to identify these active compounds, a bioguided isolation strategy would be needed. Literature data reports that polyphenols such as gallic acid and derivatives, flavonoids such as luteolin, chrysin, and apigenin, among others, induce GCL expression rather than inhibiting its activity (Panich et al., 2012, Huang et al., 2013). The second possibility (b) is less likely to happen due to cell death, as we were working with concentrations of plant extracts more than ten times lower than their maximum non-toxic concentration ($>1000\mu\text{g/mL}$). More refined experiments would be needed to attest the formation of GSH-adducts, as stated in the third possibility (c). As for the fourth and final possibility, (d) is another target that should be studied if the mechanism of GSH depletion needs to be unveiled.

Erythrina mulungu Benth. was the only extract found to increase GSH levels. Although this effect is not harmful in itself, the plant's ability to cause HDI cannot be ruled out. Safety warnings are still necessary regarding these other types of interactions that may be important in this case.

Changes in the intracellular GSH levels could also be linked to a non-functional γ -glutamyl cycle. This cycle serves as a continuous source of cysteine, which plays a key role in GSH synthesis (section 2.4.5.1). Deficiency of GGT activity could potentially lower GSH levels due to loss of cysteine (as

glutathione) in the urine. Therefore, it is possible that GSH synthesis could be impaired due to the absence of this amino acid (Lu, 2013, Chevez-Barrios et al., 2000).

In the present study, we hypothesised that reduction of GSH levels in HepG2 cells caused by the herbal extracts could be correlated with an inhibition of GGT activity. This hypothesis was also the subject of an investigation made by Jean and coworkers (2002). In their experiment, deficiency in GGT activity detected in the lung of mice resulted in lower GSH levels and, consequently, increased oxidative stress to the organ (Jean et al., 2002). On the other hand, another study suggests that depletion of GSH levels would cause an increased GGT activity as an adaptive response in order to provide more cysteine for GSH synthesis (Chinta et al., 2006).

The evaluation of GGT activity may mechanistically clarify the variation of intracellular GSH levels in HepG2 cells. The standard enzymatic assay for determination of the GGT activity is carried out by quantifying the amount of *p*-nitroaniline produced by the breakdown of GpNA in the sample. However, another enzymatic method published by Wickham and coworkers (2011) has been proposed. This new method assesses the hydrolytic activity of GGT measured by a coupled assay in which the glutamate released by the hydrolysis of the γ -glutamyl bond is oxidised by glutamate dehydrogenase, reducing NAD⁺ to NADH. In presence of diaphorase, the produced NADH reduces iodonitrotetrazolium to INT-formazan, which is a coloured product that can be detected spectrophotometrically (Wickham et al., 2011). The standard method was chosen for our *in vitro* GGT activity analysis because it is the most commonly assay used among researchers and it is not a coupled experiment like the one reported by Wickham and coworkers (2011), which is more time- and reagent-consuming.

Indeed, the reduction of GSH levels found in HepG2 cells treated with *Cordia verbenacea* A. DC., *Costus spicatus* (Jacq.) Sw., *Persea americana* Mill., *Salix alba* L., *Schinus terebinthifolia* Raddi and *Syzygium jambolanum* (Lam.) DC. turned out to be due - at least in part- by its ability to lower GGT activity. However, *Bidens pilosa* L., *Casearia silvestris* Sw., *Equisetum arvense*

L., *Lamium album* L., *Lippia sidoides* Cham., *Malva sylvestris* L., *Maytenus ilicifolia* Mart. Ex Reissek, *Solanum paniculatum* L., *Solidago microglossa* DC., *Tabebuia avellanedae* Lorentz ex Griseb., and *Vernonia polyanthes* Less, did not affect GGT activity in a significant way, so GSH levels in those cases may have been affected via other mechanisms.

We attempted the dereplication of potential GGT inhibitors in several Brazilian native species using HPTLC analysis. The flavonoid luteolin was found in *Lippia sidoides* Cham. and gallic acid was found in *Solanum paniculatum* L. Those compounds have been reported to deplete intracellular GSH levels. A study published by Balyan and coworkers (2015) demonstrated that intracellular GSH levels were significantly depleted by luteolin in the human melanoma SK-MEL-28 cell line. This was explained by an inhibition of GST activity via competitive reversible and irreversible mixed mechanisms (Balyan et al., 2015). Another study published by Locatelli and coworkers (2009) showed that gallic acid was able to deplete intracellular GSH levels in melanoma cells through inhibition of the activity of γ -Glutamyl-cysteine synthetase (Locatelli et al., 2009). Gallic acid has been also proven to inhibit GGT activity in mice (Mahajan and Mahmood, 2009).

Some herbal medicines that have been reported to affect both phase I and phase II metabolisms are also known to influence the bioavailability of drugs by modulating other targets such as P-gp. For example, the efflux of xenobiotics mediated by P-gp in intestinal cells has been reported to be affected by herbal drugs like SJW. This is due to a substrate overlap at receptor sites of P-gp caused by the consumption of herbal medicines and synthetic drugs with similar functional groups (Awortwe et al., 2014).

Several *in vitro* preclinical studies are available in the existing literature to determine if a xenobiotic is a P-gp substrate or inhibitor. Among them, the bidirectional transport assay, ATPase activity assay, and uptake/efflux assay are the most commonly used, and for such studies, Caco-2 cells have been extensively used in both the industry and in academia. Several advantages can bolster the use of this cell line for efflux studies. First, Caco-2 resembles the human intestinal barrier in morphology and polarity. It expresses numerous

proteins, including P-gp, and it is one of the most employed models in preclinical investigation to predict the gastrointestinal permeability. Second, Caco-2 is recommended by the US FDA (Food and Drug Administration) as an integral component of the Biopharmaceutics Classification System (BCS). Third, it is the most commonly used cell model in the industry to screen new chemical entities and for the prediction of DDI and HDI (Awortwe et al., 2014).

The screening of *in vitro* P-gp activity with Caco-2 cells is not devoid of drawbacks. One is the reported downregulation of the P-gp expression during cell culturing process. In addition, the P-gp expression is quite variable in this cell line which seems to be correlated with its origin and passage number (Shirasaka et al., 2008). In order to overcome these issues, several authors have proposed new ways to induce P-gp expression in Caco-2 cells.

For instance, Eneroth and coworkers (2001) overexpressed P-gp in Caco-2 cells prior to applying their use in a calcein AM extrusion screening assay. To do so, they cultured normal Caco-2 cells in media containing several concentrations of vincristine. The group concluded that an optimal level of P-gp was achieved using vincristine 25nM and that it would be suitable for their calcein AM extrusion method (Eneroth et al., 2001).

Shirasaka and coworkers (2006, 2008) managed to increase the expression of P-gp by culturing Caco-2 cells in media containing vinblastine 50nM. The mRNA concentration of P-gp increased in more than 4-fold compared to normal Caco-2 cells. This group proposed some possible reasons for the overexpression of P-gp in Caco-2 cells treated with vinca alkaloids. One possibility was that the overexpression of P-gp was mediated by the activation of hPXR. Another proposed explanation was that P-gp expression in Caco-2 is not uniform and that the vinca alkaloids treatment would have probably killed the cells with low P-gp expression, therefore acting as a selection process instead of induction. No conclusion has been reached about the correct P-gp overexpression mechanism yet (Shirasaka et al., 2006, Shirasaka et al., 2008).

To complicate matters further, studies on P-gp efflux inhibition may use different substrates. A commonly used substrate probe is digoxin, however concerns have been raised about the use of this drug due to the high variability

in the results. Jouan and coworkers (2016) carried out an *in vitro* evaluation of P-gp efflux inhibition comparing Rh-123 vs. digoxin transport. The authors concluded that Rh-123 is the most convenient substrate for characterising the effects of several P-gp inhibitors, including verapamil. Importantly, the inter-laboratory reproducibility of digoxin transport experiments was lower when compared to the results found using Rh-123. Therefore, the predictability of drug interactions could be questioned using this substrate (Jouan et al., 2016).

Thus, in our study we followed the protocol involving the overexpression of P-gp as reported by Eneroth and coworkers (2001). This resulted in an increased P-gp activity of Caco-2 cells cultured with vincristine compared to normal Caco-2, which made the conduction of our preclinical study possible. Despite this, the IC_{50} of verapamil – a reference for P-gp inhibition – in normal Caco-2 cells is not significantly different than in overexpressing Caco-2 cells after vincristine treatment (Mazzari et al., 2016).

These considerations involving cell models, suitable techniques and methodologies were crucial for the execution of the present study. As academic researchers continue to improve these aspects in order to provide better techniques for more reliable results, prediction of HDI made in early development stages will become more reliable and feasible for both industry and academia. Although most models and techniques used in the private sector are still out of reach of the average academic group, researchers in academia have been applying cutting-edge techniques to overcome the limitations of less sensitive models, thus making such studies accessible to all levels of research investment.

This is probably one of the reasons why research on HDI has considerably improved over the years (Figure 1-1), even as funding is becoming very limited in many countries. The *in vitro* assessment of the effects of herbal medicines in metabolic and transporter targets is an important step for prediction of HDI. Some examples involving our findings and the influence of intrinsic factors in the metabolism will be further discussed.

4.2 IMPLICATIONS OF HERBAL MEDICINES AND INTRINSIC FACTORS ON THE METABOLISM AND EFFLUX

In chapter 1, we showed that metabolism studies involving herbal medicines have been widely reported, especially since 1999. Since then, a plethora of herbal medicines has been the subject of studies for several targets, mainly CYP and P-gp. We also mentioned in section 1.4 that drug metabolising enzymes can be induced and/or inhibited by xenobiotics, which also encompasses herbal medicines. Some examples of documented HDI were provided in section 1.6 and, in order to map the potential HDI of the medicinal plants from RENISUS, we conducted a literature review of their effects on main metabolic and transporter targets (Table 1-2).

Besides xenobiotics, some intrinsic factors such as genetic polymorphism, disease, age, and sex could cause alterations in the expression and activity of those targets. We will integrate here these factors together with the findings of our literature review and the results of our preclinical study. We will also take into account the mix of races in Brazil which contribute to a variety of genetic polymorphisms in the country, and have been partially revealed by REFARGEN. We sought in this way to theoretically predict HDI between RENISUS medicinal plants and RENAME essential medicines. Hence, consumption of those herbal medicines could be carefully evaluated by the Brazilian physicians in the light of these considerations and the treatment that the patient is being submitted to.

4.2.1 RENISUS herbal medicines as modulators of metabolism and efflux and potential HDI with RENAME essential medicines

At this stage of our work, we can affirm that various herbal medicines have been found to affect both the metabolism and the efflux *in vitro* and *in vivo* in certain cases. Our literature review disclosed the effects of various medicinal plants that comprise RENISUS in the selected research targets. We previously mentioned in section 1.8 that in 1975 Brazil adopted a list of essential medicines named RENAME and that physicians in SUS should only prescribe medications

present in that list to the patients. Nevertheless, due to the future inclusion of more herbal medicines (apart from the 12 that are already in RENAME), the likelihood of HDI has increased.

As a step towards the implementation of herbal medicines in SUS and their safe consumption with synthetic drugs, we have thus provided some potential HDI based both on the literature review and original laboratory research findings.

4.2.1.1 Potential HDI between RENISUS herbal medicines and RENAME drugs

As previously mentioned, the expression of the main CYP isoforms found in the liver (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5/7) can be induced and/or inhibited by xenobiotics. Indeed, various RENISUS and RENAME drugs have been identified as inhibitors and/or inducers of those CYP isoforms. Therefore, it is possible that HDI could occur with the concomitant use of those drugs. We highlight here that our *in vitro* evaluation must now be clinically assessed in order to infer whether the following suggested interactions are likely to happen or not.

Beginning with CYP1A2, our literature review revealed that nine RENISUS medicinal plants were found to interfere with the activity of CYP1A2. The beta-blocker propranolol and the tricyclic antidepressant clomipramine are essential drugs extensively prescribed in Brazil. The biotransformation of propranolol and clomipramine starts with the *N*-dealkylation, governed by *S*-mephenytoin (CYP1A2) for the former and the *N*-demethylation of the side chain of the molecule of the latter to form the active metabolite desmethylclomipramine (DCIP) (Nielsen et al., 1996). Therefore, plant species that inhibit CYP1A2 such as *Eucalyptus globulus*, *Glycine max*, *Harpagophytum procumbens*, *Mentha piperita*, *Phyllanthus amarus*, and *Punica granatum* must be avoided during the treatment period with these drugs. This is, however, theoretical and in this case no reports have thus far been found in the literature.

The anticoagulant warfarin is prescribed for the treatment of venous thrombosis and pulmonary embolism. The metabolism of this drug can easily be altered by CYP1A2 inhibitors. A rare case of interaction of warfarin with

garlic (*Allium sativum*) supplements has been reported, leading to excessive bleeding (Baxter and Stockley, 2008). Other cases of HDI were detected after coadministration of *Curcuma longa*, *Glycine max*, *Harpagophytum procumbens*, *Punica granatum* and *Trifolium pratense* with Warfarin, resulting in metabolic alterations (Liu et al., 2013, Cambria-Kiely, 2002, Ramsay et al., 2005, Komperda, 2009, Heck et al., 2000).

Regarding the enzyme CYP2C9, HDI involving RENISUS and RENAME could also happen. The active metabolites E-3174, norfluoxetine, 4'-HPPH and 6-hydroxy fluvastatin, are formed through the action of CYP2C9 on the essential drugs losartan, fluoxetine, phenytoin, and fluvastatin, respectively (von Moltke et al., 1997, Scripture and Pieper, 2001, Joy et al., 2009). Concomitant consumption of those drugs along with the RENISUS medicinal plants *Allium sativum*, *Eucalyptus globulus*, *Harpagophytum procumbens*, *Mentha piperita*, *Punica granatum*, *Trifolium pratense* and *Zingiber officinale* could affect the formation of these substances because of their inhibitory effects on the enzyme.

Ibuprofen is a common medication prescribed by SUS to combat pain and inflammation. The drug is also included in the WHO Model List of Essential Medicines as a drug for pain and palliative care and as an antimigraine medicine (WHO, 2012, WHO, 2013). Ibuprofen is mostly metabolised by CYP2C9 leading to the formation of the active metabolite (S)-ibuprofen. Herbal species that are substrates for CYP2C9 may inhibit the formation of (S)-ibuprofen, potentially leading to therapeutic failure (Mo et al., 2009).

Metabolism studies with the plant species *Allium sativum*, *Eucalyptus globulus*, *Mentha piperita* and *Trifolium pratense* revealed that they are able to inhibit CYP2C19 (Foster et al., 2001, Unger and Frank, 2004). Due to the extensive use of these herbal medicines that are metabolised by CYP2C19, HDI at this level could be very frequent but so far no clinical report has been found.

Regarding CYP2D6, the popular medicinal plants *Eucalyptus globulus*, *Harpagophytum procumbens*, *Mentha piperita*, *Phyllanthus amarus*, *Punica granatum* and *Trifolium pratense* are shown to inhibit the activity of the enzyme

in liver cells (Mazzari and Prieto, 2014a). The intake of these herbal drugs may increase chances of ADRs, although no reports on HDI involving those medicinal plants and essential drugs were found in the literature.

As well as CYP2D6, our study showed that several plants like *Allium sativum*, *Momordica charantia*, *Phyllanthus amarus*, *Phyllanthus urinaria* and *Punica granatum* were able to decrease the levels and the activity of CYP2E1 in the liver (Taubert et al., 2006, Le Bon et al., 2003, Raza et al., 1996, Hari Kumar and Kuttan, 2006, Shen et al., 2008, Faria et al., 2007b, Faria et al., 2007a). As acetaminophen is also metabolised by CYP2E1, NAPQI can be also formed at this metabolic route. Therefore, consumption of these medicinal plants could reduce the formation of the toxic metabolite. For example, species of the *Phyllanthus* genus are traditionally used for conditions such as jaundice, gonorrhea, frequent menstruation, diabetes and as a pain killer (Naaz et al., 2007, Patel et al., 2011). A study revealed that *Phyllanthus urinaria* inhibits CYP2E1 activity in hepatocytes and it also attenuates acetaminophen-induced hepatotoxicity in mice. The experiment was conducted by treating a total of 37 mice with acetaminophen at a dose of 550mg/kg of body weight on day one in order to induce liver injury. The mice were then divided into two groups: the first group was treated with *Phyllanthus urinaria* extract from day two to four whereas the second group just received water. The final results indicated that the herbal drug was able to inhibit the formation of NAPQI and, consequently, prevent liver failure (Hau et al., 2009).

Among the studied CYPs, CYP3A is the most relevant family of phase I metabolism (Wienkers and Heath, 2005). Some HDI cases involving CYP3A and RENISUS medicinal plants have been described in the literature. For example, HIV-positive patients are commonly treated with the essential drug saquinavir. At the same time, dietary supplements such as garlic and/or immune system boosters such as cat's claw (*Uncaria tomentosa*) can be used to help prevent, combat and improve health. In 1998, a case report was published demonstrating that saquinavir is a substrate of CYP3A4 and that garlic (an inhibitor of CYP3A) was able to interfere with the metabolism of the

drug, leading to failures in therapy and possible drug resistance (Chen et al., 2011).

Another example of a CYP3A substrate is the HMG-CoA reductase inhibitor atorvastatin. This essential medicine is prescribed to patients with dyslipidemia to reduce levels of total cholesterol, low-density lipoprotein cholesterol (LDL), triglycerides, and very low-density lipoprotein cholesterol (VLDL) and to increase high-density lipoprotein cholesterol (HDL) levels. A study conducted in 2012 showed that the half-life of the drug was increased in rats treated with *Allium sativum* L. due to inhibition of CYP3A4 by the herbal medicine (Reddy et al., 2012).

We report for the first time the following herbal drugs to cause changes in CYP3A4 gene expression *in vitro*: *Apuleia ferrea* (Mart.) Baill., *Casearia silvestris* Sw., *Equisetum arvense* L., *Lamium album* L., *Maytenus ilicifolia* Mart. Ex Reissek, *Passiflora incarnata* L., *Persea americana* Mill, *Rhamnus purshiana* DC., *Salix alba* L., *Schinus terebinthifolia* Raddi, *Solanum paniculatum* L., *Syzygium jambolanum* (Lam.) DC. and *Vernonia polyanthes* Less. These will need to be clinically investigated to prevent such negative interactions in patients as those discussed above.

Those cases of potential HDI involving CYPs can also be explored in phase II conjugation mechanisms. According to our literature review, twenty-three RENISUS plant species were found to affect the intracellular GSH levels. Our preclinical study showed that twenty-one other plant extracts are able to significantly modulate the intracellular GSH levels in HepG2 cells.

The metabolite NAPQI, generated by the metabolism of acetaminophen, undergoes GSH conjugation which is then metabolised and eliminated from the body (Baxter and Stockley, 2008). When production of NAPQI exceeds liver stores of GSH, however, the organ is damaged due to the attachment of NAPQI to liver proteins (Alipour et al., 2013). Thus, a combined therapy of acetaminophen and herbal species that deplete GSH levels should be monitored for these herbs.

A study demonstrated that consumption of garlic extract protects hepatocytes against acetaminophen-induced GSH depletion. In order to

measure GSH levels, hepatocytes were isolated from male Sprague-Dawley rats and incubated with different concentrations of garlic extract at three different times: before, at the same time and 30 minutes after addition of acetaminophen. The results were collected at different time points (from 0 to 150 minutes) and it was observed that the intake of garlic extract is able to protect hepatocytes against acetaminophen-induced toxicity by increasing intracellular GSH levels (Anoush et al., 2009).

Consumption of garlic also seems to decrease CYP3A4 activity, thereby increasing the half-life of drugs like acetaminophen. However, GSH synthesis is stimulated by this herbal medicine (Mazzari and Prieto, 2014a). Consequently, the generated NAPQI would be promptly neutralised by GSH and its toxic effects may be unlikely to happen.

In our study, the P-gp efflux activity was also modulated by certain herbal medicines. *Achillea millefolium* was shown to inhibit P-gp whereas *Allium sativum* was shown to activate the transporter (Mazzari and Prieto, 2014a). Our preclinical study revealed that P-gp activity was affected by only four extracts, *Apuleia ferrea* (Mart.) Baill., *Rhamnus purshiana* DC., *Schinus terebinthifolia* Raddi, and *Syzygium jambolanum* (Lam.) DC.. The clinical implications associated with these findings would theoretically affect the bioavailability of drugs coadministered with those P-gp substrates, but it would need to be confirmed through future clinical studies.

HDI cases have already been reported on the P-gp level. For example, the HIV-protease inhibitor saquinavir (substrate of CYP3A4) is absorbed in the intestine via P-gp. As garlic extracts may induce P-gp efflux activity, the outcome of concomitant consumption of the herbal medicine with the drug could potentially reduce the bioavailability of the latter (Williamson et al., 2009). On the other hand, we found that *Apuleia ferrea* (Mart.) Baill., *Rhamnus purshiana* DC., *Schinus terebinthifolia* Raddi and *Syzygium jambolanum* (Lam.) DC. could successfully increase the Rh-123 concentration in Caco-2 VCR cells, an effect directly correlated to an inhibition of the P-gp efflux activity. Thus, it may be possible to increase the bioavailability of saquinavir if the drug is coadministered with these herbal extracts.

While our overview of HDI focuses on the potential harm and health risks to patients who use these herbal drugs, other studies have attempted to provide evidence of the potential benefits of HDI. For the most part, such beneficial cases are related to the effects of plant extracts on P-gp efflux activity, which could result in increased bioavailability of prescription drugs with poor oral bioavailability.

Zhang and coworkers (2007), for example, showed that curcumin from turmeric (*Curcuma longa* L.) is capable of down-regulating intestinal P-gp expression, which could then increase the bioavailability of the beta blocker celiprolol (Zhang et al., 2007). Bansal and coworkers (2008) also found that the flavonoid quercetin could increase the C_{max} of the anticancer drug irinotecan, which could in turn benefit the patients being treated (Bansal et al., 2008).

While such findings seem to be very promising examples for improved therapy using herbal medicines, each HDI requires careful consideration and a thorough review. Data collected from our literature review, for example, suggests that consumption of *Curcuma longa* L. could also decrease the expression of CYP1A2. According to Pavithra and coworkers (2009), when taken alone the drug norfloxacin has limited absorption in the gastrointestinal tract, whereas after intake of curcumin the plasma concentration of norfloxacin increases (Pavithra et al., 2009). However, norfloxacin is a well-known CYP1A2 inhibitor (Kobayashi et al., 2011). While the absorption of norfloxacin could be improved by coadministration of curcumin, therefore, their concomitant use could drastically reduce the CYP1A2 expression and compromise the metabolism of some other CYP1A2 substrates that may also have been prescribed to the patient, such as acetaminophen or propranolol. Considering these factors, it remains unclear whether such an HDI would actually benefit a patient.

The beneficial effect of the flavonoid quercetin on irinotecan can be also questioned. Although irinotecan is also metabolised by CYP3A4 (Santos et al., 2000), Choi and coworkers (2011) showed that quercetin is able to inhibit CYP3A4 activity in a concentration-dependant manner (Choi et al., 2011). As a consequence, although the plasma concentration of irinotecan is increased by

the HDI with P-gp, the effects of the drug could be exacerbated by decreased CYP3A4 activity caused by quercetin.

The suggested benefits of HDI should thus be carefully assessed. As herbal extracts contain numerous pharmacological active substances, they could affect multiple metabolic and transporter targets at the same time. Hence, one beneficial effect could be masking an undesirable one that imposes risk to the patient. Indeed, our preclinical study showed that most of the herbal medicines were able to affect more than one target. *Apuleia ferrea* (Mart.) Baill., *Rhamnus purshiana* DC., *Schinus terebinthifolia* Raddi and *Syzygium jambolanum* (Lam.) DC., for example, were able to inhibit the P-gp activity in Caco-2 VCR cells. But they were also able to modulate the CYP3A4 expression and GSH levels. Thus, it is not clear whether the use of these drugs would ultimately prove an advantage with regards to enhancing drug bioavailability, as this benefit could be compromised by the effect of herbal medicines on metabolic enzymes and other detoxification mechanisms. Nevertheless, as research produces greater knowledge of the herbal medicines' metabolic profiles, combination therapies to improve their therapeutic outcomes will be possible in the foreseeable future.

4.2.2 Intrinsic factors that impact in the regulation of metabolism and efflux

In the risk-benefit analysis of HDI, other key factors must also be taken in consideration. This includes, of course, the intrinsic factors that each patient will present. All the efforts made by using herbal medicines to improve the absorption of drugs with poor oral bioavailability could be jeopardised due to the presence of genetic polymorphisms and other factors such as disease, age, and sex of the patient. We will cover those cases and their impact on the metabolism in the following sections below.

4.2.2.1 Genetic polymorphism

Metabolism of drugs is not only affected by the coadministration of herbal medicines or other drugs. In many cases, the effects of a drug vary among subjects and these variations may not be directly correlated to the

patient's treatment. It has been found that people from different races around the globe may have a variant allele of a single gene that is responsible for producing a metabolising enzyme. This allele will codify the expression of variants of the drug metabolising enzyme, resulting in differences in the enzyme expression. For example, Caucasian and Negro populations have a proportional expression of fast and slow acetylators, whereas Asian groups have 90% fast acetylators (Shenfield, 2004).

Cases of genetic polymorphism involving metabolising enzymes and proteins have been reported. For instance, Wei-lin and coworkers (2006) reported a case of dose adjustment of the immunosuppressive drug tacrolimus because of genetic polymorphism. Tacrolimus dose regulation was required in Chinese liver transplant patients due to the presence of polymorphisms in CYP3A5, which was found to decrease the bioavailability of the drug (Wei-lin et al., 2006).

ADRs caused by genetic polymorphism involving the expression of P-gp have been also reported in the literature. Charles and coworkers (2012) reported that a patient who was receiving amlodipine for controlling a cardiovascular disorder developed a gingival hyperplasia as a result of inflammation and fibrosis, which could be correlated with the use of anticonvulsants, antihypertensive calcium antagonists, and the immunosuppressant cyclosporine. DNA analysis from a blood sample revealed that the patient presented an MDR1 3435C/T gene polymorphism, which may have modified the P-gp expression in the endothelial layers of blood vessels in the gingiva. As a consequence, this genetic polymorphism could have worsened the inflammatory response to the drug and may thus be the reason of this side effect (Charles et al., 2012).

Cases of genetic polymorphism were found among the Brazilian population, which is comprised of a plethora of ethnic groups. As stated in section 1.5.1, the assessment made by REFARGEN in the Brazilian population demonstrated that genetic polymorphism could cause some ethnic subgroups to present ADRs, leading to increased or decreased levels of drug metabolising enzymes such as CYPs and transporters.

CYP1A2, for instance, is considered to be highly inducible and polymorphic. Two polymorphisms have been found to be common among South Brazilians: the CYP1A2*1F and *1K alleles, which cause higher and decreased enzyme expression, respectively (Kohlrausch et al., 2014). Their high frequency of both alleles in Southern Brazilians, therefore, suggests that this ethnic subgroup possesses a higher risk of ADRs.

The CYP2C9*2 and *11 alleles are found in higher frequency in white Brazilians, whereas CYP2C9*3 is mostly found in browns and CYP2C9*5 in the black Brazilian population. These polymorphisms decrease the enzyme activity *in vitro* (Zhou et al., 2009), and for that reason consumption of medicinal plants such as garlic, eucalyptus, devil's claw, mentha, pomegranate, red clover, and ginger may further inhibit their function or expression, potentially causing HDI within these ethnic groups.

Approximately twenty-four variants of CYP2C19 are known. Three of them have been found by REFARGEN to exist in the Brazilian population: CYP2C19*2, CYP2C19*3, and CYP2C19*17. The alleles *2 and *3 appear not to affect the enzyme activity, whereas CYP2C19*17, which is the most prevalent, increases *in vitro* activity thereby potentially decreasing the pharmacological effects of CYP2C19 drug substrates, such as diazepam and phenobarbital (Mazzari and Prieto, 2014a).

Among the CYPs studied by REFARGEN, polymorphisms on CYP2D6 are the most present in the Brazilian population. To date, seventy-two variants of this isoform have been found in humans and, according to REFARGEN sixteen were detected in Brazilians from all researched ethnics groups: CYP2D6*1, *2, *3, *4, *5, *9, *10, *17, *29, *34, *35, *39, *41, *1XN, *2XN, and *4XN. An increase in enzyme activity was found on the alleles *1XN and *2XN, whereas a decrease of activity was found on *9, *10, *17, *29, and *41 (Cai et al., 2006). The chances of undesirable effects caused by disturbances in the bioavaioability of CYP2D6 drug substrates, such as the essential medicines atenolol and propranolol for example, are therefore elevated in these groups.

Polymorphisms on CYP2E1 in the Brazilian population have not yet been mapped by REFARGEN. CYP3A5 polymorphisms among Brazilians have

been reported with the most frequent alleles being CYP3A5*1, *3, *6 and *7. The alleles *3 and *6 decrease enzyme activity in vitro, whereas *1 and *7 showed no effects (Zhou et al., 2009). Interestingly, the allele *3 is the most common variation of this isoform in all groups in Brazil (Refargen, 2014). Since it reduces the enzyme activity, intake of herbal medicines that exert the same effect should be strongly avoided in order to prevent HDI.

Similar studies on polymorphisms in the Brazilian population of enzymes involving GSH conjugation, such as GST, have not been carried out by REFARGEN. Some reports, however, point out its existence and frequencies among this population. Rossini and coworkers (2002), for instance, reported the existence of GST polymorphisms in a group of 519 Brazilians from Rio de Janeiro. GSTM1 is involved in the detoxification of polycyclic aromatic hydrocarbons and some mutagens, whereas GSTT1 catalyses the metabolism of halomethanes by human erythrocytes. Rossini and coworkers (2002) found that the null allele, that is, no expression of the enzyme, was detected in approximately 10% of the studied population (Rossini et al., 2002). Null individuals are generally more susceptible to DNA damage by the action of GST polymorphisms like GSTM1 and GSTT1. The existence of these polymorphisms is thought to be common among multi-ethnic populations such as in Brazil. Therefore, the extensive list of plant species found to affect the GSH conjugation mechanism plus the presence of such polymorphisms could potentially increase chances of HDI among Brazilians.

REFARGEN has not yet published data about polymorphisms on UDP-glucuronosyltransferase enzymes. However, information about polymorphic UGTs and their impact on adverse drug reactions and cancer susceptibility has been reported (Guillemette, 2003). Also, no data was found about genetic polymorphisms in the Brazilian population on enzymes responsible for sulphation, methylation and acetylation conjugation mechanisms to date.

Studies have shown that the gene ABCB1, which encodes P-gp, is very polymorphic and that the pharmacokinetics of several P-gp substrates could be significantly altered (Scheiner et al., 2010). REFARGEN has identified three ABCB1 polymorphisms in the Brazilian population: 1236T (rs1128503),

2677nonG (rs2032582) and 3435T (rs1045642). Further studies on drugs that affect the regulation of ABCB1 are needed to prevent a HDI on that level in the future.

4.2.2.2 Disease

In section 1.5.2, we briefly showed that malaria-infected people could present decreased hepatic CYP activity compared to healthy subjects. Indeed, diseases can have a direct effect on both expression and activity of metabolic enzymes and transporters. For instance, a report published by Morgan (2009) showed that inflammation associated with chronic diseases can lead to the down-regulation of CYP3A4. Also, patients with secondary hepatic cancer showed lower levels of CYP1A2, CYP2C and CYP2E1 compared to the control group (Morgan, 2009).

A case report published by Lopez Galera and coworkers (2008) showed that a 45-year-old, HIV-positive woman with cirrhosis caused by a hepatitis C infection was not responding satisfactorily to the treatment of the anti-HIV drugs. The patient had no good adherence to the HIV treatment, so she was then asked whether she was making use of any other medicines, including herbal medicines. Surprisingly, the patient had been taking *Uncaria tomentosa* preparation for 2 months, probably to enhance her immune system. She was asked to cease taking the herbal medicine, and after 15 days the C_{min} values of the anti-HIV drugs were normalised. *Uncaria tomentosa* has a high inhibitory capability to CYP3A4, which causes an increase in the C_{min} values for antiretroviral agents leading to an increased risk of toxicity. The combined therapy with these two agents has been shown to be a potential risk for HIV patients (Lopez Galera et al., 2008).

Uncaria tomentosa is one of the RENISUS herbal medicines and the case report by Lopez Galera and coworkers (2008) correlates to the *in vitro* inhibitory effect in CYP3A4 reported by Budzinski and coworkers (2000). Furthermore, two herbal medicines from our preclinical study, *Lamium album* L. and *Solanum paniculatum* L., were also able to decrease CYP3A4 expression. Consumption of these herbal medicines by HIV-positive patients should, therefore, be avoided until clinical evidence proves its safety.

Besides phase I, phase II mechanisms like GSH conjugation could be impaired due to lower levels of GSH found in people with certain health conditions. A study conducted by Droge and Breitkreutz (2000) showed that the immune system works best when the GSH levels of lymphoid cells are kept within optimal levels. The study showed that some HIV-infected patients lose the GSH precursor cysteine at more levels than a non-infected person. GSH deficiency was thus observed to result in low protection of the cells against ROS, but the GSH levels in the lymphoid cells were restored with the administration of NAC (Droge and Breitkreutz, 2000). Low intracellular GSH levels have been correlated to a wide array of health conditions including neurodegenerative diseases such as Parkinson's and Alzheimer, eye diseases such as cataracts, glaucoma and macular degeneration, osteoporosis, cancer cardiovascular diseases, pulmonary diseases, cystic fibrosis and inflammatory diseases (Lomaestro and Malone, 1995, Ballatori et al., 2009).

In another relevant case report published by Senadhi and coworkers (2012), a 26-year-old man recently diagnosed with HIV was admitted to hospital presenting some symptoms that included yellow eye discoloration, dark coloured urine, and nausea. A liver function exam revealed elevated levels of the liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and serum bilirubin. Mild hepatomegaly was also detected by an abdominal computed tomography, and no signs of acute hepatitis (A, B, and C) were detected. Serum acetaminophen levels were also unremarkable. After a few appointments, the patient disclosed he was making use of the herbal medicines fucoisan, maya nut, and finger millet, which are traditionally used in some parts of Africa for HIV treatment. He was asked to discontinue the herbal HIV medications, and after 2 weeks his liver function went back to normal, and his symptoms also disappeared. The authors suggested that the GSH levels of the patient were already lowered due to the HIV infection and that the herbal medicines may have lowered those levels even further, and the self-administered herbal medicines were thus the likely cause of the patient's increased susceptibility to drug-induced hepatitis (Senadhi et al., 2012).

Such case reports raise concerns about the intake of most of the herbal medicines of our preclinical study. Herbal medicines like *Passiflora incarnata* L. or *Salix alba* L., for example, are commonly used by the Brazilian population for controlling anxiety and as anti-inflammatory respectively. Consumption of the herbal medicines that were found to deplete GSH levels in our study could, therefore, cause the same effects as those of the African ones used by the patient in Senadhi and coworkers (2012).

GSH deficiency has been associated with defects of some enzymes that take part of GSH synthesis, such as γ -glutamylcysteine ligase, glutathione synthase, GGT, dipeptidase, and 5-oxoprolinase. GGT disorders, for example, can cause central nervous system abnormalities, and cases of GGT deficiency are commonly present in cases of increased GSH concentration in the urine (glutathionuria) and in the blood plasma will be found. Glutathionuria is caused by the inability of GGT to break down GSH that is filtered and secreted in the renal tubular fluid. A study conducted with mice revealed that problems associated with GGT deficiency may cause not only glutathionuria, but also glutathionemia, growth failure, cataracts, lethargy, shortened life span and infertility (Ballatori et al., 2009).

The importance of GSH in the maintenance of the optimum state of the cells and its pivotal role in xenobiotic detoxification makes it the most important phase II conjugation mechanism. It is essential, therefore, that prescription of herbal medicines be made carefully by the physician in order to avoid more complications with the patient's health.

HDI may increase health risks among patients with preexisting diseases, such as those which may affect the P-gp expression in organs and tissues. For example, Jeynes and Provias (2013) showed that brain samples from patients with Alzheimer's disease present significant differences in P-gp expression in both the hippocampal and superior temporal parts of the brain, as compared to a normal brain (Jeynes and Provias, 2013). Jeynes and Provias (2013) also found decreased P-gp expression in the patient's inflamed intestinal epithelium, which has been previously reported to aggravate Alzheimer's disease (Blokzijl et al., 2007).

Di Rosa and Di Rosa (2014) published a case report suggesting that low P-gp levels in the BBB could be correlated to catatonia, which is a subtype of schizophrenia. Their report showed that a 20-year-old man experienced many symptoms of catatonia, such as immobility, negativism, and sweating after an overdose of loperamide. Loperamide is a P-gp substrate and has an agonistic effect on the opioid receptors. In clinical doses, loperamide is safe and P-gp prevents the drug from crossing the BBB (Di Rosa and Di Rosa, 2014).

In these cases we have described, it is possible that inhibition of the P-gp efflux activity could have also been caused by consumption of herbal medicines. In section 1.9, we showed that *Achillea millefolium* L. and *Curcuma longa* L. have been reported to inhibit the P-gp activity, and in our preclinical study, *Apuleia ferrea* (Mart.) Bail., *Rhamnus purshiana* DC., *Schinus terebinthifolia* Raddi, and *Syzygium jambolanum* (Lam.) DC. presented the same inhibitory effect. Although no clinical evidence of P-gp inhibition caused by these four has been reported yet, based on the current evidence we report here, prescription of these herbal medicines should be avoided to patients with the above conditions.

4.2.2.3 Age

Age-related changes in some metabolic enzymes and transporters have also been identified by researchers, which would contribute to possible HDI in patients with certain conditions. Ku and coworkers (2010) reported that the levels of CYPs varied in male rats aged 3, 12, 26 and 104 weeks. CYP1A1 was only detected in 3-week-old-rats, whereas CYP1A2, CYP2B1, and CYP2E1 had their maximum expression in same-age rats, although the levels significantly decreased at 12 and 26 weeks. Other CYPs like CYP2C11 and CYP3A4 have been found at low levels in aged rats (Yun et al., 2010).

Aging can also affect GSH levels. Neurodegenerative diseases like Parkinson's and Alzheimer's disease have been associated with the generation of ROS and low levels of GSH in the brain. This was demonstrated by a case study (Zhu et al., 2006) of two groups of rat subjects, one of whom was young and the other aged. The results showed that GSH was significantly reduced in the aged rat group due to the diminished activity of γ -glutamylcysteine

synthase, which is one of the enzymes responsible for GSH synthesis. From the evidence, the study concluded that the aged animals could therefore be more vulnerable to oxidative stress and, consequently, to age-related neurodegeneration diseases (Zhu et al., 2006).

Erden-Inal and coworkers (2002) also showed a correlation between aging and GSH depletion. They investigated the GSH levels in plasma collected from 176 healthy individuals, which were split into five groups according to their age: G1 (0.2 -1 years old), G2 (2-11 years old), G3 (12-24 years old), G4 (25-40 years old) and G5 (41-69 years old). The GSH levels in G1 and G5 were considerably lower than in the remaining groups (Erden-Inal et al., 2002). In the case of newborns, vulnerability to pathologies such as distress syndrome, bronchopulmonary dysplasia and periventricular leukomalacia may be a potential consequence of free radical-induced damage caused by oxidative stress (Ozsurekci and Aykac, 2016). Older people, on the other hand, could be more susceptible to previously mentioned conditions like Parkinson and Alzheimer's disease, liver disease, cancer and diabetes (Pandey and Rizvi, 2010).

An example of drug toxicity due to low GSH levels in children has been published by Sia and Chan (2006). The case report shows a situation where a 2-year-old child suffered acute liver failure due to paracetamol poisoning. The girl was admitted to hospital, presenting a high body temperature, decreased consciousness and a high heart rate. Liver function exam found the AST, ALT and ALP levels to be abnormally high, and viral studies excluded the presence of hepatitis B, C, and HIV. However, the toxicology test revealed high concentrations of paracetamol in her blood circulation. After the administration of NAC, the paracetamol level and liver enzymes began to decrease, and after 10 days of treatment, she was discharged from the hospital (Sia and Chan, 2006).

The diagnostic in Sia and Chan (2006)'s report revealed that the girl was suffering from paracetamol overdose. In section 4.2.1.1 we showed that paracetamol (acetaminophen) is first metabolised by CYPs in the liver, resulting in the formation of the toxic metabolite NAPQI, which is neutralised by GSH.

We also mentioned that some RENISUS herbal medicines may decrease both GSH levels and CYP expression, such as *Lamium album* L. and *Solanum paniculatum* L. (Table 3-5). According to Suzuki and coworkers (2010), one of the CYPs that metabolise paracetamol (CYP1A2) only reaches 80% of its normal activity in 3-year-old children or more (Suzuki et al., 2010). Therefore, use of herbal medicines in children up to 3 years old should be carefully assessed, especially since their levels of metabolising enzymes are distinct from adults, and misapplication could lead to serious health consequences.

Use of herbal medicines among children is becoming more frequent in some countries. In Germany, for example, a study published by Du and coworkers (2014) detected the use of herbal medicines among 17,450 German subjects. Approximately 5.8% were children who were using at least one herbal medicinal product, and that the highest consumption was made by children younger than 6 years old (Du et al., 2014).

Conversely, de Souza Silva and coworkers (2014) reviewed the use of herbal medicines by elderly people in North America, Europe, and Asia. The study showed that the elderly population of those areas are active costumers of herbal medicines with the most popular being chamomile, garlic, and ginger (de Souza Silva et al., 2014). Our literature review confirms that some species of chamomile, garlic, and ginger are CYP3A4 inhibitors (Mazzari and Prieto, 2014a).

Although it is not known whether herbal medicines may have a higher impact on the expression and activity of drug-metabolising enzymes and transporters among children and the elderly, consumption should be accompanied by the advice and recommendation of a health professional. More active pharmacovigilance is strongly advised for these groups.

A study on aging-related P-gp expression was conducted by Wu and coworkers (2009). They demonstrated that older mice presented increased P-gp expression; this could be a response of their organism to prevent the accumulation of toxic substances in the body (Wu et al., 2009).

Another study conducted by Warrington and coworkers (2004) suggested that age-related change in the P-gp expression is tissue-specific. In

this rat study, hepatic P-gp expression increased with age whereas renal P-gp levels were lowered in the old kidneys (Warrington et al., 2004).

Unfortunately, case reports demonstrating problems with drug therapies due to aging-related P-gp expression remain scarce and more research is needed. Although our preclinical study found that only four herbs were able to inhibit the efflux activity, their consumption by both children and elderly people should be carefully monitored.

4.2.2.4 Sex

Another aspect that must be considered in drug metabolism is the gender-related differences in the expression of CYPs, GSH, and P-gp. Sex-related changes in CYP1A2 activity in the Chinese population was investigated by Ou-Yang and coworkers (2000). Their study had 229 healthy subjects (120 men and 109 women), and the results showed that CYP1A2 activity is significantly higher in Chinese men than in women (Ou-Yang et al., 2000). As a consequence, a higher dose of the CYP1A2 drug substrate acetaminophen, for example, would be required in Chinese women in order to obtain a pharmacological effect.

Variation in GSH levels in males and females was the subject of a study published by van Lieshout and Peters (1998). Their research was conducted by collecting blood samples from 124 healthy subjects (61 females and 63 males) from different age groups. When stratified according to age group, their results did not show differences between the GSH levels between males and females (van Lieshout and Peters, 1998).

We previously mentioned in section 1.5.4 that hormones could be the reason for different levels of metabolic enzymes between men and women. Suzuki and coworkers (2006) showed that sex hormones may also be the reason for the gender-related difference in P-gp expression that was observed in rats (Suzuki et al., 2006). Also, it has been shown that female steroid hormones like oestrogens and progesterone are able to modulate the expression levels of CYP2E1 in female mouse liver, affecting the metabolism of numerous drugs (Konstandi et al., 2013).

The aforementioned study conducted by Du and coworkers (2014) also showed that consumption of herbal medicines is higher among boys aged 0 – 2 years. Those figures change in children between the ages 3 – 17, where girls are shown to use more herbal medicines than boys (Du et al., 2014).

Another study published by Stjernberg and coworkers (2006) showed the profile of herbal medicines users in the Swedish municipality of Karlskrona. In total, 1380 people were surveyed (576 males and 804 females). Approximately 20.7% of the female group admitted to using herbal medicinal products while this figure was only 16.8% among the male group (Stjernberg et al., 2006).

The lack of published studies regarding the impact of gender differences on HDI limits our discussion on this matter, but there is evidence that it is possible. Future research should be encouraged to further investigate the potential associations between HDI, patient health outcomes, and the intrinsic factors discussed (genetic polymorphism, disease, age and sex) (Sultan et al., 2015). Due to the scarcity of existing published data, the pharmacovigilance system plays an important role in collecting and providing information of patients presenting ADRs due to HDI to regulatory agencies to better identify whether any of those intrinsic factors could be contributing to the side effects.

4.2.3 Active pharmacovigilance is needed for detection of HDI

All the factors discussed here represent obstacles for an optimum drug therapy, one that is effective and with no undesirable side effects. Despite all the intrinsic factors a patient will present, the risks of intoxication or therapy failure caused by consumption of a herbal medicine could be considerably avoided if its effects on the metabolism and efflux activity were anticipated. Notwithstanding, due to the lack of regulation in this matter, HDI is mostly found at market stages and the pharmacovigilance systems are thus more likely to detect and report those cases.

Pharmacovigilance systems of many countries have been reporting cases of HDI to the WHO monitoring centre over the years. Countries like the United States, Germany, Australia, Canada, Switzerland and the United

Kingdom, for instance, were responsible for the highest number of HDI reports until 2012. In total, 27 countries reported cases of HDI to the WHO. However, there have been no cases of HDI reported from the Brazilian pharmacovigilance to the WHO to date (Mazzari and Prieto, 2014b).

Brazil implemented its pharmacovigilance system in 1999, immediately after the creation of ANVISA. Currently, the *Centro Nacional de Monitorização de Medicamentos* (National Centre for Drug Monitoring), or CNMM, uses the electronic platform *Sistema de Notificações em Vigilância Sanitária* (National Notification System for Adverse Events and Technical Complaints), or NOTIVISA, for the collection of ADRs in the country. Created in 2009, the NOTIVISA aims to receive notifications of adverse events (AE) and technical complaints cases (TC) of various products approved by ANVISA, including herbal medicines. By means of this system, this information is received by health professionals in hospitals, clinics, and laboratories, by ANVISA and other local health surveillance units, and also by professionals working in drug stores and local pharmacies. The NOTIVISA system also allows Brazilian citizens to notify any case of AE and TC, enabling a higher provision of information related to the safety of the drug (Mazzari and Prieto, 2014a).

A study published in 2010 showed that 165 AE herbal medicines were received by the pharmacovigilance centre between 1999 and 2009. Among them, only two might have occurred as a result of HDI: *Atropa belladonna* L. (Solonaceae), which was administered with acriflavine, methenamine and methylthioninium causing hepatitis in the patient, and *Pygeum africanum* Hook.f. (Rosaceae), which caused a headache due to its use in conjunction with rupatadine fumarate (Balbino and Dias, 2010). Neither *Atropa belladonna* nor *Pygeum africanum* are RENISUS species, and those HDI cases involving the plants were not reported to the WHO.

The scarcity of HDI reports in the country may be related to factors such as the lack of promotion of rational use of medicinal plants and herbal medicines as well as insufficient training of health professionals who are in the best position to detect these cases. Currently, there is a high number of professionals who can legally prescribe medicinal plants and herbal medicines

to the Brazilian population. In addition to doctors, dentists, and nurses, pharmacists, and nutritionists have the authorisation of their respective Federal Councils to prescribe these products (Camargo and Barros de Leça Pereira, 2013). However, because many of these professionals are not properly trained to safely indicate a combined herbal-synthetic drug therapy, detection of HDI by these health professionals becomes more challenging (Rates, 2001).

Detection of HDI by pharmacovigilance systems remains difficult, and in the case of the Brazilian system the few cases recorded in the country have not been properly reported to the WHO. The line between what should and should not be reported to the WHO is not well defined. For example, the *Atropa belladonna* case which resulted in hepatitis (Balbino and Dias, 2010) could be considered a case of high HDI risk when compared to the observed interaction of rupatadine fumarate with *Pygeum africanum*, which resulted in a headache. Although hepatitis is far more severe ADR than a headache, both cases should have been transmitted to the WHO. Both cases were published by Balbino and Dias (2010), but according to the report published by Skalli and Bencheikh (2012), no HDI report from Brazil was received until then (Skalli and Soulaymani Bencheikh, 2012).

It is not clear what HDI risk level the Brazilian pharmacovigilance currently deems severe enough to formalise into a report to the WHO. Thus, more attention should be given to that aspect in order to improve HDI information in the country.

5 CONCLUSIONS

In 2008, Brazil became a model country for the field of traditional medicines by defining a list of beneficial herbal medicines considered 'essential', and implementing an agenda to make the complete implementation of herbal medicines at a clinical level possible in the foreseeable future. This action taken by Brazil is a leading example of the current global trend towards the integration of herbal medicines into the healthcare system.

Our literature review has highlighted that little is yet known about native Brazilian plants. Most of the data relates to herbal medicines used worldwide, such as garlic, mint and devil's claw. We have interpreted this data within the context of the unique range of intrinsic factors including the genetic make-up of the Brazilian population and how this may cause interactions with essential drugs prescribed within the Brazilian healthcare system. It is evident that an enormous task must be undertaken to understand the effects of most local plant species in both metabolic and transporter mechanisms.

The present preclinical study provides evidence that the administration of some of RENISUS herbal medicines may possibly cause *in vitro* disturbances to metabolic mechanisms. More refined studies would be necessary to ascertain the *in vivo* and/or clinical significance of such interactions. At this point, we can suggest that active pharmacovigilance is recommended especially for the plants that affected multiple targets, such as *Apuleia ferrea* (Mart.) Baill., *Rhamnus purshiana* DC. and *Schinus terebinthifolia* Raddi, which were able to modulate all targets in our preclinical study.

Finally, it is our opinion that the Brazilian pharmacovigilance system may be underreporting HDI, and that there is a need to review the threshold of HDI risk level for reports being passed onto the WHO. Another important factor for the prevention or reduction of HDI cases is the awareness of not only health professionals, but also the regulatory authorities and consumers themselves. All these groups must realise the issues that medicinal plants and herbal medicines can cause when coadministered with synthetic drugs. The increasing cases of self-medication with medicinal plants and herbal medicines

endanger the patient's health, especially when there is no medical monitoring. Therefore, healthcare professionals including doctors, pharmacists, and nurses should encourage their patients to disclose the use of either medicinal plants or herbal medicines to prevent cases of HDI. In case of a suspected HDI, health professionals must not hesitate to report it immediately to the pharmacovigilance system. This will enable ANVISA to post a warning about risks of interactions between these drugs, which must be sent to the WHO in a more consistent manner (Skalli and Soulaymani Bencheikh, 2012).

5.1 THE WAY FORWARD

Our research group conducted for the first time some *in vitro* preclinical research using the twenty-four plant extracts from RENISUS medicinal plants to evaluate their effects in CYP3A4 mRNA gene expression, GSH levels, and P-glycoprotein activity. However, clinical studies must be carried out in order to confirm the potential HDI of RENISUS medicinal plants. According to PNPIC, Oswaldo Cruz Foundation (Fiocruz) is the Brazilian institution responsible for the creation of toxicology research centres that could be used for conducting the clinical trials with those plants.

Besides Fiocruz, numerous institutions in Brazil are involved in the implementation of herbal medicines in the SUS. A better integration of those organisations would promote a better and safer usage of herbal medicines in the country. For instance, ANVISA could develop a regulation policy to improve research on the safety of traditional herbal medicines, which could be achieved with the participation of academia in the experimental process.

REFARGEN has been doing remarkable work by mapping the genetic polymorphisms in the Brazilian population. This study should be now expanded to other groups of Brazilian living in different parts of the country in order to have a better understanding of the impact of the genetic polymorphism among the entire population.

Continued education of health professionals about the prescription of herbal medicines is strongly needed to keep the professional updated, and this could be achieved by local campaigns made by the SUS. Better awareness of

HDI could certainly help those professionals in the moment of prescription and in the identification of a potential interaction in the presence of undesirable ADRs during the patient treatment.

With these changes, it is expected that herbal medicines will be used in a more effective and rational manner, avoiding incorrect coadministration with synthetic drugs and thus helping to prevent possible cases of HDI, both in Brazil and among populations around the world.

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7 APPENDIX

7.1 INTERINSTITUTIONAL AGREEMENT BETWEEN UCL AND UNB



MEMORANDUM OF UNDERSTANDING (MOU) BETWEEN THE FUNDAÇÃO UNIVERSIDADE DE BRASÍLIA AND THE SCHOOL OF PHARMACY, UNIVERSITY COLLEGE LONDON

The *Fundação Universidade de Brasília*, herein referred to as “FUdeB”, a Brazilian federal institution of superior education, a public foundation created by Law Nº 3998 of 12/15/1961 and instituted by Decree Nº 500 of 1/15/1962 registered in the CNPJ as Nº 00.038 .174/0001-43, located on the “*Campus Universitário Darcy Ribeiro*”, 70910-900, Asa Norte, Brasília-DF, Brazil, represented in this act by its President, Prof. Ivan Marques de Toledo Camargo, Brazilian, resident and domiciled in Brasília-DF, bearer of CPF Nº 210 411 481 00 and Identity Card Nº 581564/DF, of 08/07/1980, appointed by Presidential Decree of November 14th, 2012, published in the *Diário Oficial da União* on November 16th, 2012, within the permanent jurisdiction of the respective statute, and the School of Pharmacy, University College London, a body corporate established by Royal Charter with number RC000631, having its registered office at University College London, Gower Street, London WC1E 6BT, United Kingdom, herein referred to as “UCL”, represented by its Vice-Provost (Education and International), Professor Anthony Smith have resolved to enter into the present Academic, Scientific and Cultural Memorandum of Understanding.

OBJECT

FIRST CLAUSE – The purpose of this MOU is to establish a framework within which co-operation may develop between the parties. This will occur within the context of the regulations and policies of each of the parties and subject to the availability of resources. All collaborative activities will be subject to further detailed agreements.

This MOU does not purport to constitute and/or contain a comprehensive contractual agreement between the parties but rather to record the sentiment and principles which will be applicable to the relationship between the parties

AREAS OF CO-OPERATION

SECOND CLAUSE – The parties agree to undertake programme of co-operation projects which may include:

- Visits and exchange of staff;
- Exchange of students;
- Joint organization of scientific, educational and cultural seminars, conferences, symposia and workshops;
- Co-ordinated research and joint research projects including co-operation in the fields of PhD research;
- Exchange of pedagogical experience and information.

For each co-operation project, the Parties will develop separate written agreements, setting out each Party's contribution, deliverables, responsibilities, schedule, budgets and any other items necessary for the efficient and effective achievement of such projects.

CONFIDENTIALITY

THIRD CLAUSE – The parties agree and acknowledge that both the terms of this MOU and the discussions relating to the collaborative activity are confidential and neither party will disclose them without the prior written consent of the other party.

Each party undertakes that it shall not at any time disclose to any person any confidential information concerning the activities, business or affairs of the other party except as permitted below.

Each party may disclose the other party's confidential information:

- To its employees, officers, or advisers who need to know such information for the purposes of carrying out this MOU. Each party shall ensure that its employees, officers, or advisers to whom it discloses the other party's confidential information comply with this; and
- As may be required by law, court order or any governmental or regulatory authority.

Neither party shall use the other Party's confidential information for any

purpose other than to perform its obligations under this MOU.

As appropriate, the Parties will agree terms relating to confidentiality required for specific sub-agreements.

INTELLECTUAL PROPERTY

FOURTH CLAUSE – Neither Party to this MOU shall use or refer to the name, logo or any other designation of the other Party without the prior written consent of that other Party. The Parties agree that rights to intellectual property (IPR), if any, from any collaborative projects arising out of this MOU will be dealt with by appropriate written agreements at the relevant time. For the avoidance of doubt, background IPR in any future collaboration remains the property of the contributing Parties and no rights to background IPR are conveyed by this MOU. IPR generated as a result of any joint activities or projects research shall be jointly owned by both Parties and a separate agreement on the management of IPR will be drawn up for each specific area of activity or project.

Publications resulting from joint research activities will be agreed by both Parties prior to submission. When a publication originates from one of the Parties, that Party shall acknowledge the other in any publication arising from joint activities governed by this MOU.

DESIGNATED REPRESENTATIVES

FIFTH CLAUSE – To administer the implementation of this MoU, each party shall designate a principal coordinator.

The designated principal coordinator for UCL is: *DR. JOSE MARIA PRIETO GARCIA*
[Name, address, email] *29-39 BRUNSWICK SQUARE*
LONDON, UK
EMAIL: J.PRIETO@UCL.AC.UK

The designated principal co-ordinator for FUDeB:
[Name, address, email]

VALIDITY AND TERMINATION

SIXTH CLAUSE – This MOU will come into force on the date of the signing by the two parties' authorities and will remain in force for a period of 3 years thereafter. The parties will review the agreement after two years, with a view to

preparing any future agreement. The period of validity may be extended by mutual agreement in writing of both Parties. This MOU may be terminated by either party on giving at least 60 days' written notice to the other party.

PUBLICATION

SEVENTH CLAUSE – An extract of this MoU will be published in the *Diário Oficial da União* within a period of 20 (twenty) days from the date of its signature. Such initiative and associated expenses will be the responsibility of *FUdeB*.

GENERAL PROVISIONS

EIGHTH CLAUSE – The Parties agree that the signing of this MoU does not create any legally binding obligations between the Parties.

This MOU does not bind either Party to any financial commitment.

The terms of this MOU may be amended at any time by mutual agreement in writing.

The parties agree that they will comply with the relevant rules, regulations, policies and procedures of the other organisation to the extent necessary for the purpose of the implementation and operation of this MOU.

The parties share a common commitment to applicable laws, statutes, regulations, and codes relating to anti-bribery and anti-corruption.

Any disagreement or dispute that may arise in the execution, interpretation or application of the Agreement shall be resolved by trustful negotiation between the Parties.

SIGNATURE

NINTH CLAUSE – Thus agreed, the parties sign the present MOU in 4 (four) copies of equal content, 2 (two) in the English language and 2 (two) in the Portuguese language.

For the *Fundação Universidade de Brasília* in Brasília-DF on.....(date)

.....
Ivan Marques de Toledo Camargo
President

For *University College London* in London on.....10-04-2014.....(date)



Professor Anthony Smith
Vice-Provost (Education and International)

Professor Duncan Craig
Director, School of Pharmacy

**7.2 PROJECT SENT TO CGEN AND IBAMA FOR REQUESTING
AUTHORISATION TO ACCESS THE BRAZILIAN GENETIC
HERITAGE**

**Universidade de Brasília
Faculdade de Ciências da Saúde**

**ESTUDO DE INTERAÇÕES
MEDICAMENTOSAS ENTRE AS ESPÉCIES
VEGETAIS CONSTANTES NA RELAÇÃO
NACIONAL DE PLANTAS MEDICINAIS DE
INTERESSE DO SUS (RENISUS) E
MEDICAMENTOS SINTÉTICOS**

**Proponente Brasileira: Dâmaris Silveira – Universidade de
Brasília**

**Proponente Estrangeiro: Jose Maria Prieto Garcia – UCL
School of Pharmacy**

**Pesquisador Brasileiro: André Luís Dias Araujo Mazzari – UCL
School of Pharmacy**

1) IDENTIFICAÇÃO DA PROPOSTA

Projeto: ESTUDO DE INTERAÇÕES MEDICAMENTOSAS ENTRE AS ESPÉCIES VEGETAIS CONSTANTES NA RELAÇÃO NACIONAL DE PLANTAS MEDICINAIS DE INTERESSE DO SUS (RENISUS) E MEDICAMENTOS SINTÉTICOS.

Coordenação brasileira: Dâmaris Silveira – Universidade de Brasília

Coordenação estrangeira: Jose Maria Prieto Garcia – University College of London

Proponente: Dâmaris Silveira

Período de Execução: 48 meses

2) INTRODUÇÃO

A RENISUS (Relação Nacional de Plantas de Interesse para o SUS) foi lançada em 2008 pelo Ministério da Saúde com o objetivo de selecionar espécies vegetais com atividades medicinais que possam ser integradas à Relação Nacional de Plantas Medicinais (RENAFITO), sendo disponibilizada à população pelo Sistema Único de Saúde (SUS). Para que esta meta seja alcançada, estudos farmacológicos e toxicológicos devem ser realizados para que plantas medicinais e fitoterápicos possam ser prescritos aos pacientes de forma segura.

Este projeto tem, como principal objetivo, fazer uma avaliação *in vitro* das 71 espécies vegetais presentes na RENISUS em células hepáticas e avaliar sua segurança quando a planta medicinal ou o fitoterápico for administrado com algum medicamento sintético. Estudos desta natureza são muito relevantes, uma vez que o uso de produtos naturais vem crescendo muito nos últimos anos no Brasil e no mundo. Os conceitos errôneos de que a administração de plantas medicinais é segura e isenta de efeitos colaterais podem trazer sérias consequências para a saúde pública. Um xenobiótico de qualquer natureza (como uma planta medicinal ou fitoterápico, por exemplo), uma vez administrado, será metabolizado pelas enzimas do fígado. A(s) substância(s) química(s) presente(s) naquele composto podem agir aumentando ou diminuindo a atividade ou a expressão das enzimas presentes no metabolismo de fase I (citocromo P-450) e fase II (glutathione S-transferases, UDP-glucuroniltransferases, Sulfotransferases, etc.) que podem também impactar a metabolização de medicamentos sintéticos quando estes foram concomitantemente administrados. O resultado desta interação se dá por alterações no processo farmacocinético do medicamento sintético, podendo afetar qualquer etapa do ADME (absorção, distribuição, metabolização e excreção), aumentando a toxicidade ou diminuindo a eficácia do medicamento dependendo da ação do produto natural.

Portanto, devido à necessidade de se conhecer mais profundamente a ação das espécies vegetais da RENISUS no mecanismo metabólico e identificar possíveis interações medicamentosas entre plantas medicinais e medicamentos sintéticos, este estudo toxicológico previamente aprovado e financiado pelo CNPq se faz de grande impacto para o Ministério da Saúde no processo de implementação da RENISUS e para futuros projetos envolvendo plantas medicinais e fitoterápicos no SUS.

2.1 Plantas Medicinais

Plantas medicinais e fitoterápicos são amplamente utilizados no mundo e estão também tornando-se um motivo de preocupação, principalmente em países em desenvolvimento onde muitos são utilizados como tratamento primário para doenças com AIDS, devido às suas interações com drogas convencionais.[1] O consumo de plantas medicinais cresceu de 2.5% entre pacientes em 1990 para 12.1% em 1997, sendo portanto considerado a segunda maior forma de medicina complementar e alternativa utilizada no mundo. Ásia, Índia e África são exemplos onde plantas medicinais vêm sendo utilizadas com sucesso durante séculos.[2] Devido ao conceito de que plantas medicinais são consideradas “naturais” e “sem efeitos colaterais”, são necessários esclarecimentos maiores junto à população sobre os riscos do consumo de plantas medicinais, devido aos seus numerosos compostos presentes em seus respectivos extratos com atividade farmacológica e também com certos níveis de toxicidade. A utilização indiscriminada de plantas medicinais e fitoterápicos para suposta prevenção de doenças é alvo também de alerta para uma implementação urgente de um programa global de uso racional dos mesmos.[3]

2.2 Interações Medicamentosas entre plantas medicinais e medicamentos sintéticos

A subestimação dos efeitos adversos de plantas medicinais podem ser explicados por alguns fatores, como a falta de informação dos pacientes sobre a segurança destes produtos, grande variedade de plantas medicinais no mercado com qualidade e indicações diversas, carência de estudos sobre efeitos colaterais por consumo de plantas medicinais, etc. A origem destes efeitos adversos pode ser uma indicação de interação medicamentosa surgida pelo consumo concomitante de uma planta medicinal ou fitoterápico e um medicamento convencional, ao invés de uma reação causada por um composto isolado. Tanto a farmacocinética como a farmacodinâmica do medicamento sintético podem ser alteradas em casos de ingestão deste com uma planta medicinal ou fitoterápico.[4]

Interações farmacocinéticas estão diretamente envolvidas nos processos de absorção, distribuição, metabolização e excreção (ADME) do medicamento. A absorção pode ser alterada por diversas razões, como mudanças no pH intestinal, por alterações na motilidade, no efluxo ou na captação de transportadores. Estas alterações pode causar distúrbios no

mecanismo de distribuição, interferindo com a ligação do medicamento com as proteínas plasmáticas, causando distúrbios nos processos metabólicos de fase I (funcionalização) e fase II (conjugação) e também no processo de excreção devido à modificação do pH da urina e da modulação de transportadores em hepatócitos e túbulos renais.[5]

As interações farmacodinâmicas podem causar mudanças nas respostas farmacológicas que podem resultar em um aumento ou completa inibição da atividade da substância farmacológica da droga coadministrada. Os efeitos podem se dar por uma interação antagônica (competitiva ou não-competitiva), sinérgica ou aditiva. Em resumo, os potenciais resultados de uma interação medicamentosa entre plantas medicinais ou fitoterápicos e o medicamento sintético podem levar a alterações farmacocinéticas e/ou farmacodinâmicas do último, causando mudanças na sua eficácia e toxicidade. [5]

Em tempos onde a utilização de plantas medicinais se populariza e integração das mesmas no SUS torna-se cada vez mais presente e necessária, uma atenção maior deve ser oferecida às possíveis interações medicamentosas em níveis metabólicos para que se tenha um maior entendimento em como o consumo de produtos naturais e fitoterápicos se processa do ponto de vista farmacológico.[6]

2.3 Metabolismo de xenobióticos

Xenobióticos são compostos não presentes naturalmente em organismos vivos, como um medicamento, por exemplo.[7] Quando um xenobiótico é ingerido, o processo metabólico é iniciado por diversas enzimas para que o composto seja eliminado e o organismo detoxificado. Estas enzimas metabolizadoras de drogas estão presentes por diversas partes do corpo, sendo a maioria encontrada no fígado. As reações de biotransformação são divididas em duas fases: metabolismo de fase I (ou reações de funcionalização) e metabolismo de fase II (reações de conjugação). As enzimas metabolizadoras de drogas possuem um papel importante neste processo, por converterem o xenobiótico em um composto mais hidrossolúvel, permitindo sua eliminação do organismo. [8]

No metabolismo de fase I, a estrutura do xenobiótico é modificada pela adição de grupos funcionais apropriados para que então os mesmos sejam submetidos à conjugação por ácido glicurônico, sulfato ou outra molécula altamente polar, tornando o metabólito mais solúvel em água.[9] Este mecanismo é caracterizado por processos de oxidação e redução com a inserção de grupos funcionais (OH, SH ou NH₂, por exemplo) ao substrato.[10] Este processo envolve o citocromo P450 e suas enzimas microssomais, como CYP1, CYP2, CYP3, CYP4 e CYP7, localizando-se prevalentemente na membrana do retículo endoplasmático liso. O metabolismo de fase II engloba enzimas como as sulfotransferases (SULT), UDP-glicuronosiltransferases (UGTs), DT-diaforase ou NAD(P)H: quinona oxirredutase (NQ), ou NAD(P)H: menadiona redutase (NMO), epóxido

hidrolases (EPH), glutathione S-transferases (GSTs) e N-acetiltransferases (NAT).[11] Os derivados hidroxilados produzidos por enzimas presentes no metabolismo de fase I poderão ser conjugados para facilitar tanto a excreção biliar ou renal.[12]

Identificado em 1998, o receptor de pregnanos X (PXR) é também conhecido por receptor esteroide e xenobiótico (SXR) e ainda por receptor ativado por pregnanos (PAR). É um dos componentes da superfamília de receptores nucleares [13]. Assim será denominado pela observação de que altas concentrações de pregnanos poderiam atuar como seus ligantes naturais [14].

Este fator de transcrição exerce função crucial na homeostase de metabolização e eliminação de endobióticos e xenobióticos por meio da regulação de enzimas do citocromo P450 - especialmente a CYP3A4 e proteínas transportadoras como as proteínas resistentes a múltiplos fármacos (Multidrug resistance protein- MDR1) e a (Multidrug-resistance-associated protein - MRP2) [15,16]. Está intimamente envolvido na homeostase energética e nos processos inflamatórios [17,18].

Para exercer suas funções, o PXR, após ligar-se ao ligante, dimeriza-se ao ácido cis-9-retinoico (RXR) e liga-se a sítios específicos de ligação ao DNA, chamados de elementos responsivos (ER6, DR3 e DR4) presentes na região promotora dos genes da CYP [19,20].

A atividade transcricional do PXR pode ser modulada por diversos ligantes como fármacos, poluentes ambientais e ainda fitoterápicos [18].

Os ligantes podem ter pesos moleculares que variam de 300 Da como clotrimazol até mais de 800 Da como a rifampicina. Sendo assim, fatores como o peso molecular, a forma dos ligantes, e a quantidade de interações, hidrofóbicas, principalmente a interação aromática, e as ligações de hidrogênios parecem ser determinantes para a ligação ao receptor nuclear PXR [21].

2.4 Plantas medicinais no Brasil

O Brasil possui a maior variedade genética de plantas do mundo, mas somente cerca de 55.000 espécies estão catalogadas. O comércio de plantas medicinais no Brasil é um dos mais lucrativos do planeta. Em 1996, por exemplo, 25% dos lucros de indústrias farmacêuticas brasileiras foram originados de fitoterápicos.[22] Em 2011 o mercado de fitoterápicos movimentou cerca de R\$ 1,1 bilhão no Brasil, representando um aumento de 13% em relação ao ano anterior [23]

Devido ao vasto uso do conhecimento tradicional de plantas medicinais no país, se fez necessário a implementação de políticas públicas para melhorar e aumentar os recursos para pesquisa neste campo. Para isso, o Ministério da Saúde Brasileiro publicou em 1982 publicou o Programa de Pesquisa em Plantas Medicinais e Fitoterápicos (PPPM) para atender às necessidades do Sistema Único de Saúde (SUS), sob supervisão da então

Central de Medicamentos (Ceme).[24,25] O objetivo da PPPM era submeter plantas medicinais selecionadas de acordo com seu uso tradicional a estudos farmacológicos, como estudos pré-clínicos, estudos clínicos e toxicológicos.[26] As espécies foram selecionadas de acordo com suas respectivas atividades terapêuticas seguindo o formulário de proposição de plantas para estudos farmacológicos. Nesta etapa do programa, 21 espécies foram selecionadas de acordo com suas atividades farmacológicas, como por exemplo analgésicas, antipiréticas, sedativas, diuréticas, antiparasitárias, expectorantes, antidiabéticas, etc.[25] Após dois anos, devido aos avanços positivos do programa, uma nova lista foi publicada contendo agora 60 espécies para estudo. Em 1986, a lista foi novamente atualizada e o número de espécies então subiu para 74. No ano de 1988, 7 espécies já possuíam os resultados finais de estudos farmacológicos publicados: *Allium sativum*, *Mentha piperita*, *Maytenus ilicifolia*, *Phyllanthus niruri*, *Mikania glomerata*, *Ageratum conyzoides* and *Passiflora edulis*. Após dez anos de existência, o PPPM foi praticamente interrompido devido à crise econômica que se instalava no país, impactando diretamente os investimentos em pesquisa. Durante praticamente toda a década de 90 pouco se avançou neste campo e o PPPM acabou por não concretizar seu principal objetivo que consistia na produção e distribuição de plantas medicinais e fitoterápicos com qualidade e segurança para a população.[25]

2.5 A implementação de novas políticas e de novos programas de pesquisa em plantas medicinais no Brasil

O ano de 1999 foi marcado pela criação da Agência Nacional de Vigilância Sanitária (ANVISA) com o objetivo de promover a saúde na população brasileira, controlando a produção e comercialização de produtos (incluindo plantas medicinais e fitoterápicos) e serviços em diversos níveis.[27] Devido à falta de sucesso do PPPM, fazia-se necessária a implementação de novos projeto para novamente impulsionar o desenvolvimento da pesquisa em plantas medicinais no país. Em 2006, o Governo Federal aprovava a Política Nacional de Práticas Integrativas e Complementares (PNPIC) no SUS, atendendo portanto às recomendações da OMS para a promoção do uso racional de plantas medicinais[24,28].

A chamada Medicina Complementar do PNPIC envolve basicamente a medicina tradicional chinesa, homeopatia, plantas medicinais e fitoterapia, hidroterapia e medicina antroposófica. O PNPIC apresenta semelhanças com os objetivos propostos pelo PPPM, como por exemplo a implementação da fitoterapia no SUS e o estabelecimento de uma política de fundos para pesquisa em plantas medicinais no país com foco na biodiversidade brasileira. Para tornar as propostas do PNPIC concretas, o Ministério da Saúde aprovou em 2009 o Programa Nacional de Plantas Medicinais e Fitoterapia (PNPMF), coordenando assim todas as medidas adotadas pela PNPIC[24].

Um dos principais objetivos da PNPIC é a criação da Relação Nacional de Plantas Medicinais e Fitoterápicos (RENAFITO). Esta lista contemplaria plantas medicinais e fitoterápicos a serem oferecidos aos pacientes do SUS e que atendam aos seguintes critérios:

- Serem utilizadas na atenção básica;
- Espécies nativas ou exóticas adaptadas;
- Com registro na ANVISA;
- Com maior número de evidências de segurança e eficácia;
- Distribuição por biomas brasileiros;
- Espécies da flora brasileira não ameaçadas de extinção (IN/2008-MMA); e
- Capacidade de produção no país.

2.6 A Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS)

Como discutido anteriormente, as plantas medicinais para serem incorporadas à RENAFITO devem seguir os critérios estabelecidos já mencionados. Estudos etnofarmacológicos realizados nas cinco regiões do Brasil indicaram as plantas medicinais mais utilizadas em programas de fitoterapia para as doenças prevalentes em cada uma delas. Este estudo deu origem em 2008 à Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS).[29] A lista contempla 71 espécies nativas e exóticas adaptadas que servem como referência para estudos de eficácia e segurança das mesmas para que estas plantas medicinais possam ser incorporadas à RENAFITO e então serem produzidas e dispensadas aos usuários do SUS.

3) JUSTIFICATIVA

Para atender aos anseios da Política Nacional de Práticas Integrativas e Complementares (PNPIC) em relação à elaboração da Relação Nacional de Plantas Medicinais e Fitoterápicos, o Governo Federal aprovou a Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS) para que sejam definidas as espécies que poderão ser futuramente incorporadas ao SUS. Considerando os riscos apontados sobre possíveis interações medicamentosas entre plantas medicinais e medicamentos sintéticos em nível metabólico, um estudo desta natureza se faz de grande relevância para a saúde pública do país, uma vez que os profissionais do SUS estarão aptos a prescreverem terapias combinadas com fitoterápicos e medicamentos sintéticos. Além disso, o estudo será um alerta aos usuários de plantas medicinais e fitoterápicos quando ao seu uso racional, principalmente quando outra terapia medicamentosa estiver sendo adotada pelo paciente.

Desta forma, o estudo dos efeitos de espécies listadas na RENISUS no metabolismo e os riscos potenciais de ocorrência de interações medicamentosas se faz de grande importância tanto para os pacientes quanto ao SUS e à PNPIC.

4) OBJETIVOS: GERAL E ESPECÍFICOS

Considerando que:

- Existe um grande aumento do uso de plantas medicinais e fitoterápicos no Brasil devido aos falsos conceitos de segurança por serem de origem natural;
- Há uma carência de estudos sobre os efeitos das espécies contidas na RENISUS no metabolismo
- O risco de interações entre planta medicinal ou fitoterápico com medicamentos sintéticos, podendo resultar na falha terapêutica do paciente é real e de impacto na saúde pública
- A PNPIIC estabelece como meta a criação da RENAFITO, e que para isso se faz necessário o estudo completo de eficácia e segurança das espécies contidas na RENISUS.

Os objetivos são:

GERAL:

Fazer uma avaliação *in vitro* de espécies vegetais presentes na RENISUS quanto à segurança .

ESPECÍFICOS:

- 1) Realizar estudos *in vitro* em células HepG2 para avaliação dos efeitos de extratos de espécies presentes na RENISUS no metabolismo de fase I (citocromo P450)
- 2) Realizar estudos *in vitro* em células HepG2 para avaliação dos efeitos de extratos de espécies presentes na RENISUS na fase II (glutathione e UGT)
- 3) Realizar estudos *in vitro* em células CaCo2 para atividade da glicoproteína-P.
- 4) Avaliar a ocorrência de agonismo ou antagonismo em Receptores de Pregnano X.

5) IDENTIFICAÇÃO DAS INSTITUIÇÕES/UNIDADES ONDE SERÃO REALIZADAS CADA ETAPA DO PROJETO COM DISCRIMINAÇÃO DAS RESPECTIVAS ESTRUTURAS

Universidade de Brasília (UnB): Laboratório de Produtos Naturais e Laboratório de Controle da Qualidade, em parceria com pesquisadores do Departamento de Botânica:

O **Laboratório de Produtos Naturais (LPN)** da Universidade de Brasília fornecerá os extratos brutos vegetais que farão parte da Coleção de extratos do grupo de Pesquisa. O LPN realizará todas as etapas envolvendo as plantas, até a elaboração dos extratos brutos vegetais que serão testados no projeto. No **Laboratório de Controle da Qualidade (LCQ)** serão

realizados os processos analíticos (cromatográficos e espectrométricos), necessários para obtenção de um perfil químico dos extratos obtidos.

Os testes de toxicidade *ex vivo* serão realizados no **Centro de Pesquisa em Doença Celiaca**, capacitado com toda a estrutura necessária para os ensaios com diversas linhagens celulares.

No **Laboratório de Farmacologia Molecular (FARMOL)** serão realizados os testes de agonismo e antagonismo em PXR.

Os pesquisadores Sueli Maria Gomes e Christopher Willian Fagg são os botânicos que atuarão na identificação do material botânico. A coleta será realizada conforme descrito no item Material e Métodos.

O **Herbário UB** conta com ferramentas para coleta das plantas no campo, prensas para herborização, estufa de campo e sala de estufa para a desidratação do material coletado.

Os laboratórios da Universidade de Brasília contam com corpo técnico disponível, equipamentos e insumos básicos necessários para a realização da pesquisa conjunta. Acesso à biblioteca, periódicos Capes e outras fontes de pesquisa estão disponíveis a todos os estudantes e pesquisadores.

UCL School of Pharmacy

O Dr. Jose Maria Prieto Garcia é Diretor do curso de mestrado em Farmacognosia da UCL School of Pharmacy e possui vasta experiência com plantas medicinais e testes *in vitro* com diversas linhas celulares. O doutorando André Luís Dias Araujo Mazzari foi bolsista do mais bem reconhecido programa de bolsas de estudo da Europa (Chevening Scholarship) para realização do seu mestrado em farmacognosia na UCL School of Pharmacy em 2011. Mazzari possui dois anos de experiência também com plantas medicinais e linhas celulares, que foram objetos de sua tese de mestrado.

Na School of Pharmacy, serão realizados testes *in vitro* com as linhas celulares HepG2 (células de hepatoma humano) e CaCo2 (adenocarcinoma de cólon humano) para avaliação da toxicidade dos extratos e suas respectivas concentrações máximas não-tóxicas (CMNT). As células HepG2 serão utilizada para avaliar a atividade dos extratos nas enzimas responsáveis pelo metabolismo de fase I e fase II. As células CaCo2 serão utilizadas para avaliação da atividade dos extratos sobre a glicoproteína-P.

Equipe Brasileira:

Pesquisador	Instituição	-Atividades a serem desenvolvidas
Dra Dâmaris Silveira Dra Yris Maria Fonseca Dra Pérola Oliveira Magalhães	LCQ/FS/UnB LPN/FS/UnB	Obtenção das amostras e preparação dos extratos, análises cromatográficas e espectrométricas
Dra Yanna Karla de Medeiros Nóbrega	CPDC/FM/UnB	Testes de toxicidade <i>ex vivo</i>

Dr Luiz Alberto Simeoni	FARMOL/FS/UnB	Testes de agonismo/antagonismo em PXR
Dra Sueli Maria Gomes Dr Christopher William Fagg	IB/UnB FCE/UnB	Identificação botânica e catalogação

Equipe Britânica:

Pesquisador	Instituição	-Atividades a serem desenvolvidas
Dr. Jose Maria Prieto Garcia André Luís Dias Araujo Mazzari	UCL School of Pharmacy	Análises cromatográficas, testes <i>in vitro</i> com as células HepG2 e CaCo2.

6) MATERIAL E MÉTODOS

6.1) Obtenção do material botânico

O material vegetal será obtido da seguinte forma:

Quando disponível em Farmácias Vivas ou Programas de Fitoterapia vinculados à Secretaria de Saúde Municipal ou Estadual, o material vegetal será coletado para utilização *in natura* ou após secagem em estufa com circulação forçada de ar, de acordo com o preconizado pelo Programa fornecedor da amostra.

Quando não for possível a obtenção de amostra da espécie vegetal por meio dos programas supracitados, a droga vegetal será adquirida de fornecedores de insumos vegetais ou ervanarias.

Todas as amostras serão analisadas farmacobotanicamente de forma a assegurar a identidade das mesmas. As espécies serão identificadas pelos taxonomistas que participam do projeto, com auxílio da literatura especializada. Será confeccionada uma exsicata testemunha, a ser incorporada no acervo do UB.

Os extratos serão obtidos conforme preconizado no Formulário Fitoterápico da Farmacopeia Brasileira (FFFB) [30], na RDC 10/10, que trata notificação de drogas vegetais [31], na Lista de Medicamentos Fitoterápicos de Registro Simplificado [32], ou conforme protocolo de obtenção das Farmácias Vivas ou Programas de Fitoterapia.

Os extratos obtidos serão submetidos à concentração à secura, utilizando técnica adequada ao solvente extrator utilizado (por exemplo, liofilização para extratos aquosos, evaporação sob vácuo para extratos etanólicos) e armazenados a 30 °C até a sua utilização.

Damaris

6.2) Obtenção do perfil cromatográfico e/ou espectrométrico

Os extratos obtidos serão submetidos a técnicas cromatográficas clássicas (CCD e CLAE-DAD) no sentido de serem obtidos perfis cromatográficos. Eluentes e método de análise serão definidos e utilizados de acordo com o extrato obtido. A espectrometria na região do infravermelho e/ou na região do ultravioleta também poderá ser utilizada no sentido de analisar o extrato obtido.

6.3) Avaliação toxicológica dos extratos vegetais por Sulfarodamina B em células HepG2 e CaCo2

Esta etapa será realizada pela equipe britânica assim que as amostras chegarem à UCL School of Pharmacy.

A metodologia utilizada para este ensaio é a Sulfarodamina B.[33] Esta metodologia é comumente aplicada para avaliar citotoxicidade em diversas linhas celulares. O mecanismo baseia-se na captação do corante aminoxantina (SRB) que possui carga negativa por aminoácidos básicos nas células. O número de células viáveis é detectado de acordo com as que possuem coloração rosa. Quando as células são lizadas, a cor torna-se mais intensa e a absorbância aumenta.[33]

Após contagem, as células são inseridas em placas de 96 poços e os extratos são então adicionados. Após o período de incubação, as células são fixadas com 100µl de solução de ácido tricloroacético (TCA) 40% p/v em água deionizada. As placas são incubadas a 4°C por 1 hora e rinsadas 5 vezes com água destilada. São adicionadas às células fixadas com TCA 100 µl de solução de SRB (SRB 0.4% em 0,1% de ácido acético) e as mesmas são deixadas em temperatura ambiente por 1 hora. Após, as placas são rinsadas 4 vezes com ácido acético 1% para remover o corante e as placas são então secas pelo ar ambiente durante 12 horas.

Após a secagem das placas, as proteínas coloridas pelo SRB são solubilizadas pela adição de 100µl de solução 10mM de tampão Tris em cada poço. As placas são agitadas por 30 segundos e a densidade óptica (DO) detectada a 492nm em um leitor de placas de 96 poços. A percentagem de mudança no crescimento das células é calculada da seguinte forma:

% de células viáveis = $[(DO \text{ (amostra)} - DO \text{ (branco)}) / (DO \text{ (controle)} - DO \text{ (branco)})] \times 100$

6.4) Avaliação de glutatona (GSH) intracelular em células HepG2

Glutatona (Fig.1) é um tripeptídeo não usual formado por 3 aminoácidos (Glutamina, Cisteína e Glicina) encontrados no córtex, medula, citosol, mitocôndria, moléculas, sangue e em grande parte no fígado. A glutatona protege o corpo removendo potenciais compostos tóxicos eletrofílicos, como radicais livres, peróxidos e agentes alquilantes.[34,35]

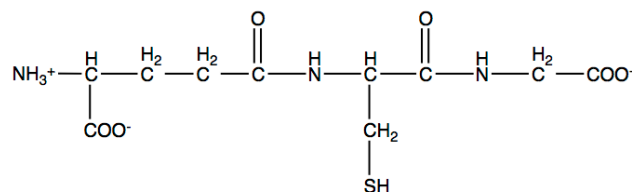


Figura 1. Estrutura molecular da glutatona

A análise de glutatona intracelular baseia-se no sistema de reciclagem pela glutatona redutase e NADPH. O composto DTNB (ácido 5,5'-ditiobis-2-nitrobenzóico) reage com GSH produzindo TNB (5'-tio-2-ácido nitrobenzóico) e GSSG (forma oxidada da glutatona). A enzima glutatona redutase irá catalisar a redução do GSSG para GSH em presença de NADPH (Fig. 2). O quantidade de TNB formada é proporcional à quantidade total de glutatona tanto na forma oxidada quanto reduzida (GSH + GSSG), sendo a quantidade total determinada verificando a absorbância das amostras em comparação com os padrões.[35]

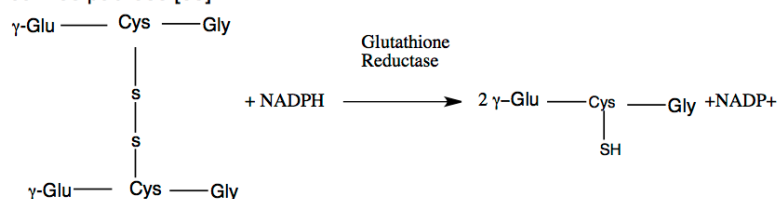


Figura 2. Formação de molécula de glutatona por ação da enzima glutatona redutase em presença de NADPH

Após contagem das células HepG2 (4×10^4 células/poço), as mesmas são colocadas em placas de 96 poços e incubadas a $37^\circ\text{C}/5\%\text{CO}_2$ por 24 horas. Após, os extratos e o controle positivo butionina sulfoxamina (BSO) são adicionados às células de acordo com as concentrações máximas não-tóxicas previamente determinadas pela análise com SBR e as placas incubadas por mais 24 horas. Posteriormente, o meio de cultura é aspirado e as células são rinsadas com 200µl de tampão fosfato-salino. São adicionados então 50µl de solução 0.1% de Triton X-100 às células e as placas são agitadas por 2 minutos. 25µl de cada poço são então transferidos para outra placa de 96 poços e reservados para análise de proteínas totais. Nos pratos de análise de glutatona, são então adicionados mais 25µl de solução de ácido salicílico 5% v/v e as placas agitadas por mais 2 minutos. Após, 25µl de

padrões de referência (glutathione reduzida) são adicionados em diferentes concentrações (100mM, 50mM, 25mM, 12.5mM, 6.25mM, 3.125mM, 1.5625mM e 0.78125mM) nas placas. 125µl da solução tampão de glutathione (7.5ml de tampão fosfato-salino + 3µl de glutathione redutase 500UI + 1ml de solução de NADPH 2,39mM + 1ml de solução de DTNB 0.01M) e as absorbâncias de cada poço são lidas a cada 30 segundos por 5 minutos a 405nm. O total de GSH é calculado de acordo com o slope dos padrões e das amostras.

6.5) Avaliação de proteína total intracelular em células HepG2

Após a análise de glutathione, são adicionadas às placas de proteínas que foram previamente reservadas 25µl de albumina sérica bovina (BSA) em diferentes concentrações (1mg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml) para curva padrão. A análise de proteína total é realizada de acordo com o protocolo do fabricante do Bio-Rad kit. Brevemente, 25µl do reagente A' são adicionadas em cada poço e, posteriormente, 200µl do reagente B são também inseridos nos mesmos. As placas são deixadas em temperatura ambiente por 15 minutos e as absorbâncias lidas a 595nm.[36] A quantidade total de proteína é calculada de acordo com a curva-padrão com BSA e então dividida pelo valor de GSH encontrado nas amostras para que os resultados finais sejam apresentados em µM of GSH/mg de proteína.

6.6) Avaliação da indução de CYPs em células HepG2

Os xenobióticos (dentre eles as plantas medicinais e fitoterápicos) possuem a capacidade de influenciar a atividade dos citocromos, causando uma alta regulação da transcrição dos seus respectivos genes. Este fenômeno tem como principal consequência o aumento da atividade enzimática dos citocromos, causando alterações na farmacocinética do medicamento sintético administrado, implicando portanto em interações medicamentosas.[37]

Brevemente, as células HepG2 (1.0×10^6 células/poço) são inseridas em uma placa de 12 poços e incubadas por 24 horas. Após, o meio de cultura é removido e substituído por outro sem soro bovino e a placa é incubada por mais 24 horas. Os extratos são adicionados às células e as placas incubadas por um período entre 24h-120h para avaliar o curso de tempo de cada expressão de CYP RNAm. Ao fim do tratamento, as células foram coletadas e o RNA total foi quantificado utilizando TRIzol (kit de extração de RNA) de acordo com o protocolo do fabricante. Em seguida, os primers de genes específicos são produzidos para ampliar as regiões dos CYPs a serem analisados e os DNAs competidores são gerados utilizando o kit de construção do DNA competitivo. Após a síntese do DNAc, uma série de PCR competitivos foram produzidas fazendo-se diluições do DNAc para se obter as diferentes isoformas dos citocromos (CYP1A, CYP3A, CYP 2E1, etc). O produto do PCR de cada CYP é então analisado por sequência direta

com cada primer. O grau de indução é calculado em relação às células de controle não tratadas.[38]

6.7) avaliação *in vitro* da citotoxicidade dos extratos em células HeLa

Com o objetivo de definir a viabilidade das células HeLa expostas ao tratamento com os extratos vegetais preparados, será utilizado o ensaio colorimétrico segundo o método do 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT; SIGMA-ALDRICH®). O anel de tetrazólio presente no sal brometo de MTT, de coloração amarelada, é clivado por desidrogenases presentes na mitocôndria das células viáveis, formando cristais de formazana, de coloração azulada [39]. Portanto, é considerado que a quantidade de células viáveis é proporcional à quantidade de cristais de formazana formada. No ensaio, com exceção dos poços utilizados para o controle do teste (branco), todos os outros poços das placas serão semeados com 30.500 células HeLa em meio de cultura DMEM. O volume final será de 100 µL. Após incubação de 24 horas a 37 °C e 5% de CO₂, serão adicionados 10 µL de MTT (5 mg/mL em solução salina tamponada com fosfato (PBS)) em todos os poços das placas, e as mesmas serão incubadas por 4 horas a 37°C e 5% de CO₂. Para a dissolução do sal de formazana, o meio de cultura será retirado dos poços e 100 µL de solução reveladora de MTT [HCl (Sigma) 0,04 M em isopropanol absoluto (Vetec)] serão adicionados. As placas serão deixadas sob agitação por 30 minutos à temperatura ambiente e a absorbância será medida a 570 nm em leitor de placas Beckman Coulter DTX 800. Os resultados serão expressos como a porcentagem de células viáveis nos poços tratados com os extratos em relação aos controles (100%). Três experimentos independentes serão realizados em triplicata para cada condição.

6.8) Ensaio de transativação (transfecção transitória por eletroporação e ensaio de gene repórter)

O gene repórter consiste de um promotor ligado ao gene repórter de interesse no qual a região promotora liga-se aos fatores de transcrição. Para evitar a interferência de fatores endógenos de células de mamíferos e ainda avaliar o efeito de ligantes isoladamente sobre o LBD do PXR, nos experimentos realizados neste trabalho serão utilizados vetores quiméricos contendo o DBD do fator de transcrição de leveduras GAL-4.

O método de eletroporação será utilizado para a transfecção transiente. Esse método consiste na aplicação de pulso de corrente elétrica a uma suspensão de células para gerar poros na membrana plasmática e nuclear e assim permitir a entrada do DNA plasmidial de interesse.

Para o ensaio da transfecção, células HeLa serão coletadas por meio da centrifugação (2.000 rpm por 5 minutos) e ressuspensas em solução

PBS contendo 0,1% de dextrose e 0,1% de cloreto de cálcio, para obtenção de uma concentração de 10^7 células/mL. 500 µL da solução de células serão transferidas para uma cubeta e submetidas à eletroporação (Gene Pulser II - Bio-Rad®) nas condições de 240 mV e 950 µF. Após a eletroporação, as células serão transferidas para o meio de cultura, distribuídas em placas de 12 poços (Corning®) (1 mL por poço) e tratadas. Para transfecções com pM-hPXR, as células serão tratadas com veículo (vcl) (EtOH:DMSO 1:2), e com os controles positivos rifampicina 10^{-5} M, clotrimazol 10^{-5} M e 150 µg/mL de erva de São João (*Hipericum perforatum*) ou com os extratos obtidos das drogas vegetais em estudo em diferentes concentrações e mantidas por 24 horas de incubação a 37 °C e CO₂ 5%. Após o período de incubação, o meio de cultura será retirado e a cada poço será adicionado 100 µL tampão de lise 1X (Promega®).

6.8.1) Cálculo da taxa de ativação da transcrição

A taxa de ativação da transcrição é a razão entre atividade (leitura da luciferase) das células tratadas com os agonistas (controle positivo) ou extratos e a atividade (leitura da luciferase) das células tratadas com o veículo (vcl) (EtOH:DMSO 1:2). Convencionando a taxa de ativação das células tratadas com veículo igual a 1 (um).

Taxa de ativação = $\frac{\text{Leitura da luciferase (ligante sintético ou natural ou extrato)}}{\text{Leitura da luciferase (células tratadas com veículo)}}$

Leitura da luciferase (células tratadas com veículo)

7) CRONOGRAMA DE EXECUÇÃO

A execução do projeto de pesquisa está prevista para ser iniciada em abril de 2014, com duração de 36 meses.

Etapa 1: Obtenção e processamento do material botânico (secagem, pulverização, obtenção dos extratos)

Etapa 2: Avaliação da citotoxicidade dos extratos em células HepG2 e CaCo2

Etapa 3: Análise dos níveis de glutatona e proteína em células HepG2 após contato com os extratos

Etapa 4: Análise dos níveis de citocromos em células HepG2 após contato com os extratos (protocolo em desenvolvimento)

Etapa 5: Avaliação da atividade da glicoproteína-P em células CaCo2 após contato com os extratos (protocolo em desenvolvimento)

Etapa 6: Avaliação do agonismo/antagonismo em PXR

Etapa7: Obtenção do perfil cromatográfico

Etapa 8: publicação dos resultados e finalização e entrega de relatório final

Etapa	Período (em número de meses)								
	0	6	12	18	24	30	36	42	48
1									
2									
3									
4									
5									
6									
7									
8									

8) INSTITUIÇÃO FIEL DEPOSITÁRIA CREDENCIADA PELO CGEN

Exsicatas do material botânico serão depositadas no Herbário da UnB (UB)

9) INSTITUIÇÕES DE DESTINO DAS AMOSTRAS, ONDE OCORRERÁ O ACESSO AO PATRIMÔNIO GENÉTICO

A **UCL School of Pharmacy** receberá amostras de extratos brutos e frações de interesse, na quantidade necessária para o desenvolvimento da parte do projeto que lhes cabe.

Curador: o derivado vegetal ficará sob responsabilidade do Dr. Jose Maria Prieto Garcia

Endereço: 29-39 Brunswick Square, Londres, Reino Unido

Post Code: WC1N 1AX

Telefone: +4402077535800

e-mail: j.prieto@ucl.ac.uk

10) RESULTADOS ESPERADOS

Além da contribuição para o conhecimento sobre espécies da biodiversidade brasileira quanto ao seu potencial farmacológico e quanto ao grau de toxicidade das mesmas em humanos, este projeto, permitirá o fortalecimento tanto de cada um dos grupos envolvidos quanto dos laços de cooperação envolvendo os pesquisadores desse projeto.

Os resultados encontrados serão de grande relevância para as pesquisas farmacológicas que estão sendo desenvolvidas com as espécies da RENISUS. Este estudo fornecerá os dados toxicológicos das espécies vegetais da RENISUS, além de orientar os prescritores do SUS quanto às possíveis interações medicamentosas das mesmas com outros medicamentos sintéticos que são também prescritos nos hospitais públicos

do país. Consumidores de fitoterápicos também podem ser beneficiados caso haja uma maior orientação farmacêutica, principalmente para os profissionais de farmácia de manipulação, quanto aos riscos de interações medicamentosas devido ao consumo de fitoterápicos em terapia combinada com medicamentos sintéticos. Finalmente, o presente estudo irá auxiliar na correta implementação da Política Nacional de Práticas Integrativas e Complementares (PNPIC) quanto à elaboração da Relação Nacional de Plantas Medicinais e Fitoterápicos (RENAFITO).

11) INDICADORES DE AVALIAÇÃO, ACOMPANHAMENTO E PRODUTOS ESPERADOS

A avaliação do projeto é feita periodicamente pelo grupo acadêmico da UCL School of Pharmacy, por meio de encontros regulares com o supervisor e por seminários organizados pelo departamento de ciências farmacêuticas da instituição. O acompanhamento também é realizado anualmente pelo CNPq através do formulário de avaliação de doutorado no exterior, na plataforma Carlos Chagas.

Ao final do projeto, além da apresentação da tese de doutorado do bolsista André Luís Dias Araujo Mazzari, os dados do projeto serão publicados em periódicos científicos brasileiros e estrangeiros. Será também mantida a continuidade dos trabalhos em conjunto entre a UCL School of Pharmacy e a Universidade de Brasília para a produção de novos projetos de pesquisa, estabelecendo, portanto, um vínculo vitalício entre ambas as instituições.

12) FONTES DE FINANCIAMENTO

Ciência sem Fronteiras / CNPq (processo CNPQ 201327/2012-0): O projeto e os recursos foram aprovados pelo CNPq na modalidade Doutorado Pleno no Exterior para o doutorando André Luís Dias Araujo Mazzari de acordo com o processo acima informado.

Recurso aprovado para o subprojeto: R\$206.559,61

Financiamento de outras fontes será buscado durante a execução do projeto.

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7.3 MATERIAL TRANSFER AGREEMENT

Jose Prieto MTA-14-199
Cachira

MATERIAL TRANSFER AGREEMENT – MTA to be used when shipping genetic heritage samples for non-commercial research purposes

The Material Transfer Agreement (MTA) was established to monitor shipments of genetic heritage existing under *in situ* conditions, within the national territory, on the continental shelf and in the exclusive economic zone, or maintained under *ex situ* conditions, intended for Brazilian or foreign research institutions based on the following principles:

- Acknowledgment that the exchange of genetic heritage between research institutions in the field of biology and related areas, based in Brazil or abroad, is of vital importance to increase knowledge of Brazilian biodiversity;
- The need to ensure compliance with the provisions of the Convention on Biological Diversity, especially national sovereignty over biodiversity, prior informed consent and sharing of benefits arising from the use of genetic heritage.

Sending Institution

Name: Fundação Universidade de Brasília (FUnB)

Address: *Campus Universitário Darcy Ribeiro*, 70910-900, Asa Norte, Brasília-DF, Brazil

Information on the representative of the Institution

Name: Prof. Ivan Marques de Toledo Camargo

ID (type, number, and issuing agency): CPF Nº 210 411 481 00 and Identity Card Nº 581564/DF, of 08/07/1980,

Position of legal representative of the Sending Institution: Professor, FUnB Legal representative

Legal document assigning authority to the legal representative: (attach a copy) Presidential Decree of November 14th, 2012, published in the *Diário Oficial da União* on November 16th, 2012.

Receiving Institution

Name: UCL School of Pharmacy

Address: 29-39 Brunswick Square, WC1N 1AX London, UK

Information on the legal representative of the Institution

Name: Prof Duncan Craig.

ID (type, number, and issuing agency): *N/A*

Position of legal representative of the Receiving Institution: *DIRECTOR OF UCL SCHOOL OF PHARMACY*

Legal document assigning authority to the legal representative: (attach a copy)

Project/Agreement in question:

Regarding the "Study of the potential herb-drug interactions between RENISUS plant species and synthetic drugs" application to CGEN (Conselho de Gestão do Patrimônio genético).

The signatory institutions, through their duly established representatives, bearing in mind the provisions of the convention on Biological Diversity, Provisional Act No. 2,186-16, dated August 23, 2001, Decree No. 3,945, of September 28, 2001, as amended by Decree No. 4,946 of December 31, 2003, and Genetic Heritage Management Council Resolution No. 20, of June 29, 2006, undertake to use the sample(s) of the genetic heritage components transferred among themselves pursuant to the following conditions:

1. The received material must only be used by the receiving institution for noncommercial scientific research purposes.

2. In cases of any subsequent wish to make use of the samples of the genetic heritage components transferred under this MTA for the purposes of bioprospection, technological development, or the request of a patent, the Receiving Institution shall undertake to so inform the Sending Institution, which shall in turn inform the Genetic Heritage Management Council or an institution accredited under the terms of Article 11(IV)(e) of Provisional Act No. 2,186, dated August 23, 2001.
3. Undertaking the activities mentioned in the previous paragraph without complying with the relevant legal provisions, and in particular without prior authorization from the Genetic Heritage Management Council, is prohibited.
4. Samples of genetic heritage components may not be transferred to third parties by the Receiving Institution unless a new MTA has first been signed between the original Sending Institution and the new Receiving Institution, in accordance with the provisions of Resolution No. 20, 2006.
5. Receiving Institutions shall abide by the terms of the MTA and shall not be considered providers with respect to the material received.
6. Any publication resulting from the use or study of shipped samples of genetic heritage components shall expressly acknowledge the origin of the material and credit the Sending Institution, to whom a copy of the publication in question must also be sent.
7. The signatory institutions shall cooperate, on mutually agreed terms, in capacity building and technology transfer, to promote the conservation and sustainable use of biological diversity, as provided for in Provisional Act No. 2.816-16, dated August 23, 2001.
8. The Sending Institution is wholly responsible for identifying and properly packing the material, and for complying with specific shipment procedures related to biological risk assessment and for the containment of the organism or material transferred, observing all relevant official recommendations, international standards and specific legislation of the Receiving Country.
9. The Receiving Institution commits itself to:
 - a. not claiming any intellectual property rights over the genetic heritage components or parts thereof transferred under the MTA, without prior access authorization issued by the Genetic Heritage Management Council;
 - b. informing the Sending Institution, in writing, of any adverse effects observed when handling the genetic heritage components under the MTA.
10. Failure to comply with the procedures set forth in this Agreement shall subject offenders to the penalties established in existing legislation.
11. The competent forum for settling disputes among institutions with respect to this MTA shall be the head office of the original Sending Institution.
12. The commitments related to the material transferred under this Agreement shall remain valid for an indefinite period of time, regardless of whether or not the Agreement has been renewed.

Having agreed with all the above provisions, the representatives of the Receiving Institution and of the Sending Institution hereby sign this Agreement, in three identical copies, each equally authentic, with equal legal effect.

Model of standard Warning Label to be attached to the outside of the package containing the shipped sample of a Genetic Heritage Component. When appropriate, a label in English, Spanish or French shall also be attached.

ATTENTION!

**Sample of Brazilian Genetic Heritage
CONTAINS BIOLOGICAL MATERIAL OF NO COMMERCIAL VALUE**

Shipment in accordance with Genetic Heritage Management Council Resolution No. 20, of June 29, 2006 (Provisional Act No. 2186-16/ 2001).

Documents which must accompany this shipment:

1. Where the Receiving Institution is based abroad, copy of the Authorization granted by the Genetic Heritage Management Council or the institution it has accredited.
2. Specification of the type and quantity of the sent material.
3. In cases where a Special Authorization on Access and Shipment has been issued, (i) a copy of the MTA, OR (ii) Export Licence issued by IBAMA

<http://www.mma.gov.br/port/cgen>

Model of standard Identification Label to be attached to the outside of the package containing a sample of a Genetic Heritage Component when returning to the sender. When appropriate, a label in English, Spanish or French shall also be attached.

ATTENTION!

**RETURN of a Sample of Brazilian Genetic Heritage
BIOLOGICAL MATERIAL OF NO COMMERCIAL VALUE.**

In accordance with Article 15 of Genetic Heritage Management Council Resolution No. 20, of June 29, 2006.

<http://www.mma.gov.br/port/cgen>

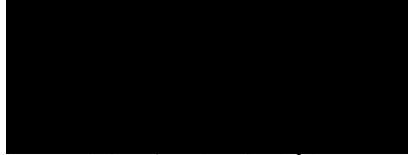
Place: UCL School of Pharmacy

Date: 25th February 2012

Anne Lane

Dr Anne Lane
Authorised UCL Signature

Representative of the Receiving Institution:



Representative of the Sending Institution:

7.4 AUTHORISATION TO ACCESS THE BRAZILIAN GENETIC

HERITAGE GIVEN BY CGEN/CNPQ



6223768071168241

AUTORIZAÇÃO DE ACESSO E DE REMESSA DE AMOSTRA DE COMPONENTE DO PATRIMÔNIO GENÉTICO nº 010295/2014-3

O CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO - CNPq, credenciado pelo Conselho de Gestão do Patrimônio Genético (CGEN/MMA), por meio da Deliberação CGEN nº 246, de 27 de agosto de 2009, para autorizar instituições nacionais, públicas ou privadas, que exerçam atividades de pesquisa e desenvolvimento nas áreas biológicas e afins, a acessar e remeter amostras de componente do patrimônio genético para fins de pesquisa científica sem potencial de uso econômico, neste ato representado pelo seu Diretor de Ciências Agrárias, Biológicas e da Saúde, nos termos da Portaria CNPq nº 104/2011, autoriza a instituição abaixo qualificada a acessar e remeter amostras de componentes do patrimônio genético.

Instituição: UNIVERSIDADE DE BRASILIA - UNB

CNPJ: 000.381.740/0001-43

Representante Legal: JAIME MARTINS DE SANTANA

Cargo/Função: Decano (Pró-Reitor) de Pesquisa e Pós-Graduação

CPF: 308.595.651-49 **RG:** 358372

Projeto: ESTUDO DE INTERAÇÕES MEDICAMENTOSAS ENTRE AS ESPÉCIES VEGETAIS CONSTANTES NA RELAÇÃO NACIONAL DE PLANTAS MEDICINAIS DE INTERESSE DO SUS (RENISUS) E MEDICAMENTOS CONVENCIONAIS

Coordenador do Projeto: Damaris Silveira

CPF: 464.405.696-00 **RG:** 2430447 - SSP / DF

Finalidade do projeto: A RENISUS (Relação Nacional de Plantas de Interesse para o SUS) foi lançada em 2008 pelo Ministério da Saúde com o objetivo de selecionar espécies vegetais com atividades medicinais que possam ser integradas à Relação Nacional de Plantas Medicinais (RENAFITO), sendo disponibilizada à população pelo Sistema Único de Saúde (SUS). Para que esta meta seja alcançada, estudos farmacológicos e toxicológicos devem ser realizados para que plantas medicinais e fitoterápicos possam ser prescritos aos pacientes de forma segura. Este projeto tem, como principal objetivo, fazer uma avaliação in vitro das 71 espécies vegetais presentes na RENISUS em células hepáticas e avaliar sua segurança quando a planta medicinal ou o fitoterápico for administrado com algum medicamento sintético. Estudos desta natureza são muito relevantes, uma vez que o uso de produtos naturais vem crescendo muito nos últimos anos no Brasil e no mundo. Os conceitos errôneos de que a administração de plantas medicinais é segura e isenta de efeitos colaterais podem trazer sérias consequências para a saúde pública. Um xenobiótico de qualquer natureza (como uma planta medicinal ou fitoterápico, por exemplo), uma vez administrado, será metabolizado pelas enzimas do fígado. A(s) substância(s) química(s) presente(s) naquele composto podem agir aumentando ou diminuindo a atividade ou a expressão das enzimas presentes no metabolismo de fase I (citocromo P-450) e fase II (glutathione S-transferases, UDP-glucuroniltransferases, Sulfotransferases, etc.) que podem também impactar a metabolização de medicamentos sintéticos quando estes foram concomitantemente administrados. O resultado desta interação se dá por alterações no processo farmacocinético do medicamento sintético, podendo afetar qualquer etapa do ADME (absorção, distribuição, metabolização e excreção), aumentando a toxicidade ou diminuindo e eficácia do medicamento dependendo da ação do produto natural. Portanto, devido à necessidade de se conhecer mais profundamente a ação das espécies vegetais da RENISUS no mecanismo metabólico e identificar possíveis interações medicamentosas entre plantas medicinais e medicamentos sintéticos, este estudo toxicológico previamente aprovado e financiado pelo CNPq se faz de grande impacto para o Ministério da Saúde no processo de implementação da RENISUS e para futuros projetos envolvendo plantas medicinais e fitoterápicos no SUS.

Amostras a serem acessadas:

Grupos Taxonômicos: Todos as espécies contidas na Relação Nacional de Plantas de Interesse ao SUS (RENISUS): *Achillea millefolium*, *Allium sativum*, *Aloe* spp (*Aloe vera* ou *Aloe barbadensis*), *Alpinia* (*Alpinia zerumbet* ou *Alpinia speciosa*), *Anacardium occidentale*, *Ananas comosus*, *Apuleia ferrea* = *Caesalpinia ferrea*, *Arrabidaea chica*, *Artemisia absinthium*, *Baccharis trimera*, *Bauhinia* spp (*Bauhinia affinis*, *Bauhinia forficata* ou *Bauhinia variegata*), *Bidens pilosa*, *Calendula officinalis*, *Carapa guianensis*, *Casearia sylvestris*, *Chamomilla recutita* = *Matricaria chamomilla*, *Chenopodium ambrosioides*, *Copaifera* spp, *Cordia* spp (*Cordia curassavica* ou *Cordia verbenacea*), *Costus* spp (*Costus scaber* ou *Costus spicatus*), *Croton* spp (*Croton cajucara* ou *Croton zehntneri*), *Curcuma longa*, *Cynara scolymus*, *Dalbergia subcymosa*, *Eleutherine plicata*, *Equisetum arvense*, *Erythrina mulungu*, *Eucalyptus globulus*, *Eugenia uniflora* ou *Myrtus brasiliana*, *Foeniculum vulgare*, *Glycine max*, *Harpagophytum procumbens*, *Jatropha gossypifolia*, *Justicia pectoralis*, *Kalanchoe pinnata* = *Bryophyllum calycinum*, *Lamium album*, *Lippia sidoides*, *Malva sylvestris*, *Maytenus* spp (*Maytenus aquifolium* ou *Maytenus ilicifolia*), *Mentha pulegium*, *Mentha* spp (*M. crispata*, *M. piperita* ou *M. villosa*), *Mikania* spp (*Mikania glomerata* ou *Mikania laevigata*), *Momordica charantia*, *Morus* spp., *Ocimum gratissimum*, *Orbignya speciosa*, *Passiflora*

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spp (Passiflora alata, Passiflora edulis ou Passiflora incarnata), Persea spp (P. gratissima ou P. americana), Petroselinum sativum, Phyllanthus spp (P. amarus, P. niruri, P. tenellus e P. urinaria), Plantago major, Plectranthus barbatus = Coleus barbatus, Polygonum spp (Polygonum acre ou Polygonum hydropiperoides), Portulaca pilosa, Psidium guajava, Punica granatum, Rhamnus purshiana, Ruta graveolens, Salix alba, Schinus terebinthifolius = Schinus aroeira, Solanum paniculatum, Solidago microglossa, Stryphnodendron adstringens = Stryphnodendron barbatimam, Syzygium spp (S. jambolanum ou S. cumini), Tabebuia avellanedae, Tagetes minuta, Trifolium pratense, Uncaria tomentosa, Vernonia condensata, Vernonia spp (Vernonia ruficoma ou Vernonia polyanthes), Zingiber officinale

Tipo de material/quantidade de amostras: O tipo de amostra dependerá da espécie. A quantidade aproximada será: folhas, sumidades floridas ou partes aéreas: 500 g; caule, raiz ou bulbos: 800 g; frutos ou sementes: 600 g

Local de depósito de subamostra: UNIVERSIDADE DE BRASÍLIA

Equipe do projeto: DAMARIS SILVEIRA / CPF 464.405.696-00
ANDRE LUIS DIAS ARAUJO MAZZARI / CPF 090.900.047-69
YANNA KARLA DE MEDEIROS NOBREGA / CPF 020.423.464-62
YRIS MARIA FONSECA / CPF 045.789.276-52
PEROLA DE OLIVEIRA MAGALHAES / CPF 248.940.408-32
LUIZ ALBERTO SIMEONI / CPF 043.388.018-08
FRANCISCO DE ASSIS ROCHA NEVES / CPF 186.580.591-20
RICCARDO PRATESI / CPF 000.079.941-68
CHRISTOPHER WILLIAM FAGG / CPF 694.231.131-68
JOSE M PRIETO / CPF ...

Validade da Autorização: 15/05/2014 a 01/05/2018

A instituição acima qualificada deverá enviar ao CNPq, por meio do Coordenador do Projeto, relatório anual sobre o andamento do projeto de pesquisa, nos termos do Decreto nº. 4.946/2003. O roteiro para confecção do relatório está disponível em <http://www.cnpq.br/web/guest/relatorio-de-atividades>. Os relatórios devem ser enviados ao CNPq em meio eletrônico, para o endereço apg@cnpq.br e, preferencialmente, em formato .pdf.

Esta autorização está vinculada às informações, declarações e termos de compromisso firmados pelo coordenador do projeto e pelo representante legal, constantes do Processo nº 010295/2014-3. Atividades de acesso aos conhecimentos tradicionais associados, de acesso e de remessa de componente do patrimônio genético com finalidade comercial, aplicação industrial, bioprospecção ou desenvolvimento tecnológico não estão autorizadas.

Caso seja identificado uso econômico de produto ou processo, passível ou não de proteção intelectual, originado das amostras de componente do patrimônio genético acessado no âmbito desta autorização, a instituição beneficiada se compromete a adotar as providências cabíveis, nos termos da legislação vigente, junto ao CGEN/MMA.

Se ocorrer coleta de espécie não autorizada ou não identificada, deverá ser observado o que consta no Decreto nº 6.514, de 22/07/2008, no que refere à flora e fauna, e em particular sobre espécies ameaçadas de extinção ou de endemismo estrito.

A remessa de amostra de componente do patrimônio genético deverá ser precedida da assinatura do Termo de Transferência de Material (TTM) ou do Termo de Responsabilidade para Transporte de Amostra de Componente do Patrimônio Genético (TRTM). A remessa para instituições nacionais está isenta de autorização prévia. Contudo, a remessa para instituições sediadas no exterior depende de autorização prévia do CNPq, nos termos das resoluções do CGEN 15/2004 e 20/2006. Os modelos dos termos, assim como as citadas resoluções, estão disponíveis em <http://www.cnpq.br/web/guest/remessa-e-transporte> e devem ser enviados ao CNPq em meio eletrônico para o endereço apg@cnpq.br, preferencialmente em formato .pdf. Ainda, para a remessa de componente do patrimônio genético para instituição sediada no exterior, deverá ser solicitada ao Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA, por meio de formulário específico e mediante a apresentação de TTM ou TRTM, licença de exportação complementar a autorização de remessa, especialmente quando se tratar de remessa de espécies constantes nos Anexos da Convenção sobre o Comércio Internacional das Espécies da Flora e Fauna Selvagens em Perigo de Extinção (Cites).

Brasília, 15 de Maio de 2014

Marcelo Marcos Morales

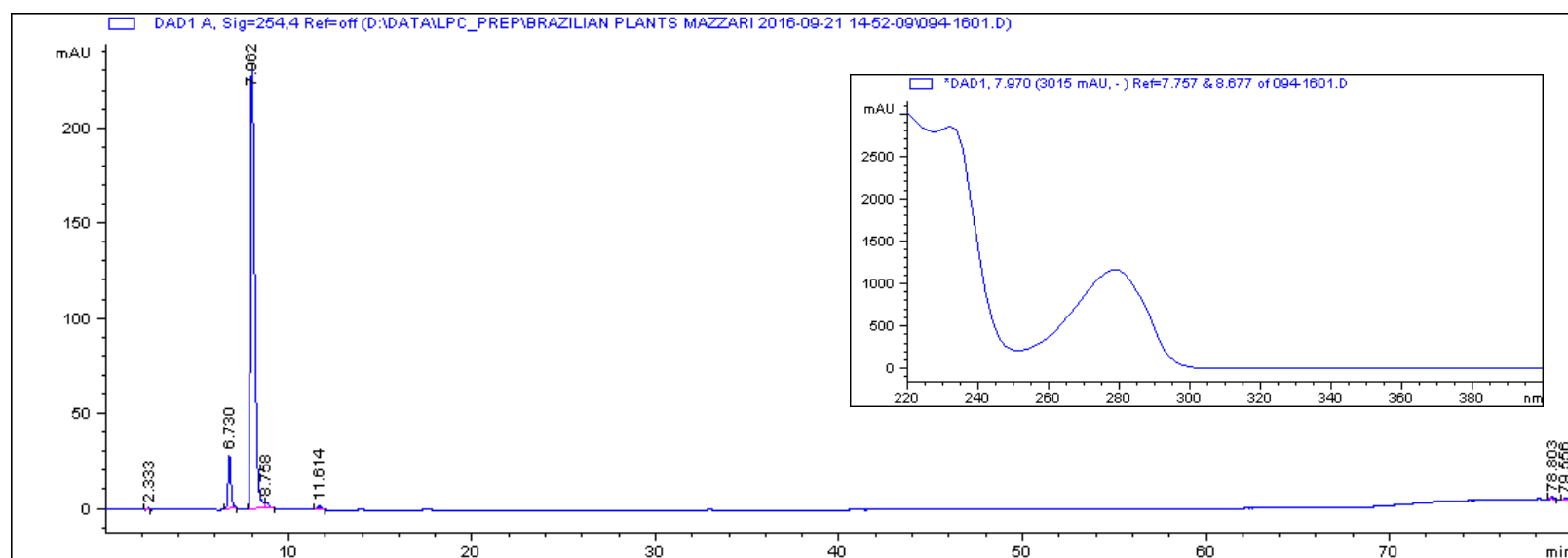
Diretor de Ciências Agrárias, Biológicas e da Saúde

Para visualizar a versão digital da Autorização de Acesso e de Remessa de Amostra de Componente do Patrimônio Genético, V.Sa. poderá utilizar a ferramenta disponibilizada pelo CNPq para esse fim na página <http://servicosweb.cnpq.br/visualizador/> e informar o número do protocolo 6223768071168241 para recuperá-la do banco de dados do CNPq, ou poderá selecionar o arquivo salvo em seu computador (em formato PKCS7). V.Sa. pode também usar outro aplicativo disponível no mercado capaz de reconhecer arquivos no padrão PKCS7 para fazer a

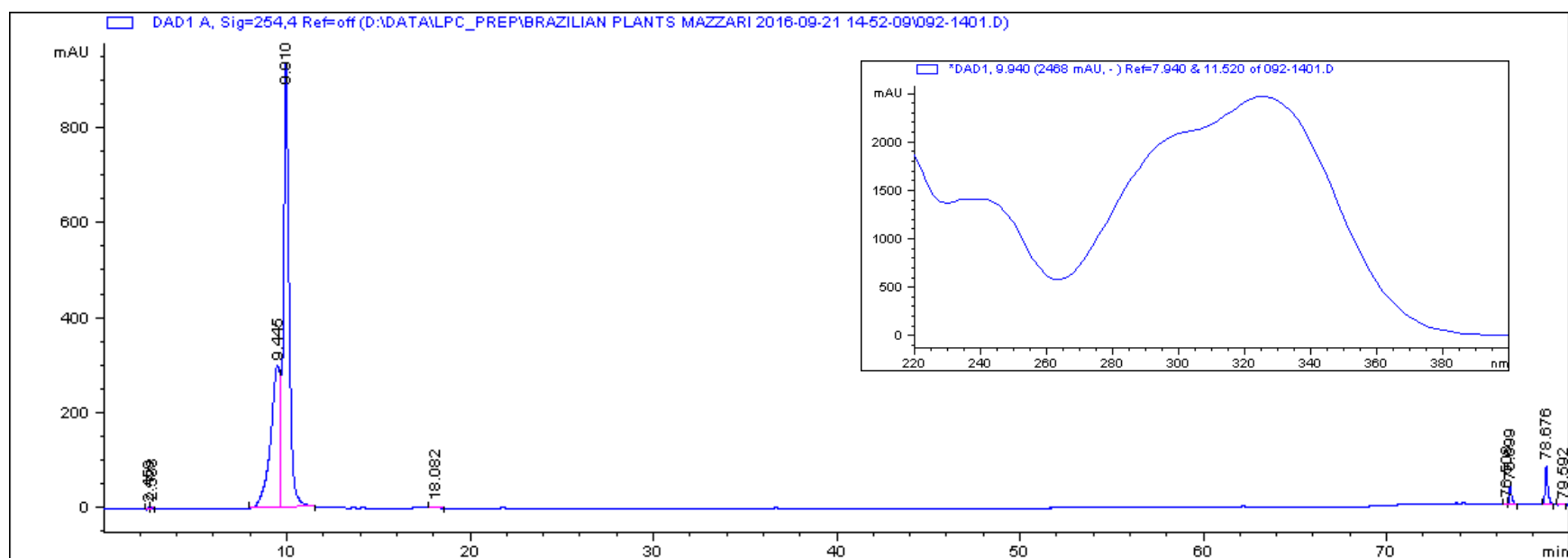
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7.5 HPLC ANALYSIS

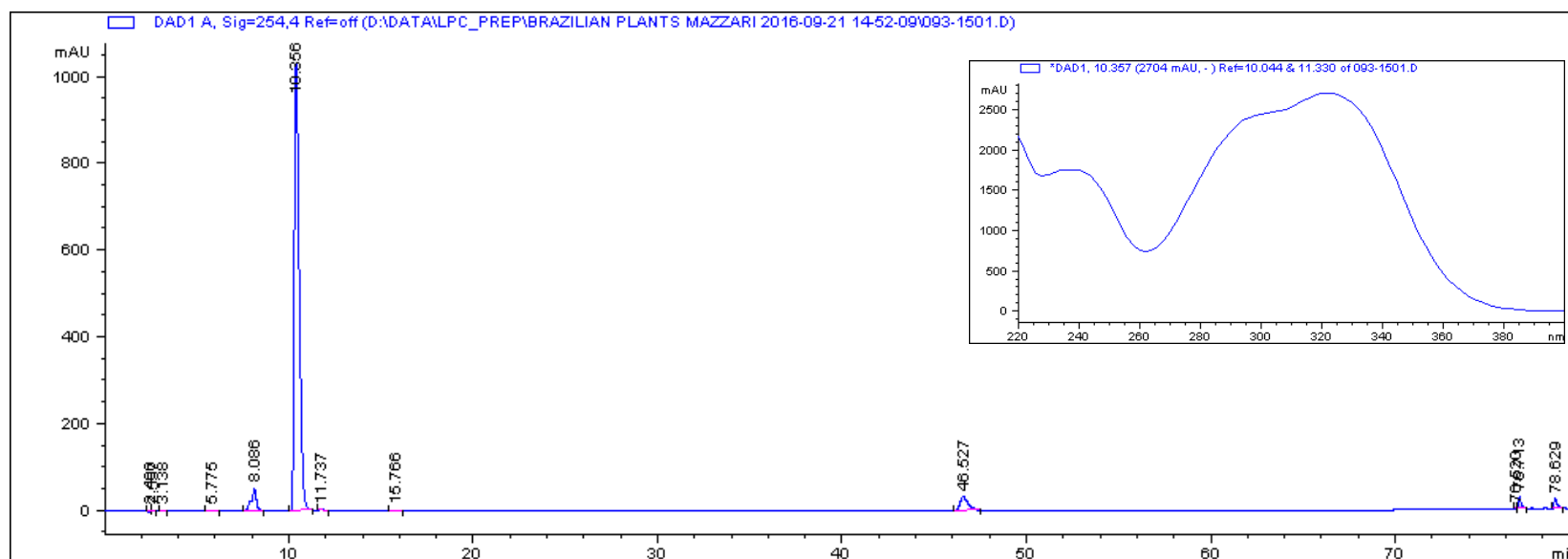
Catechin



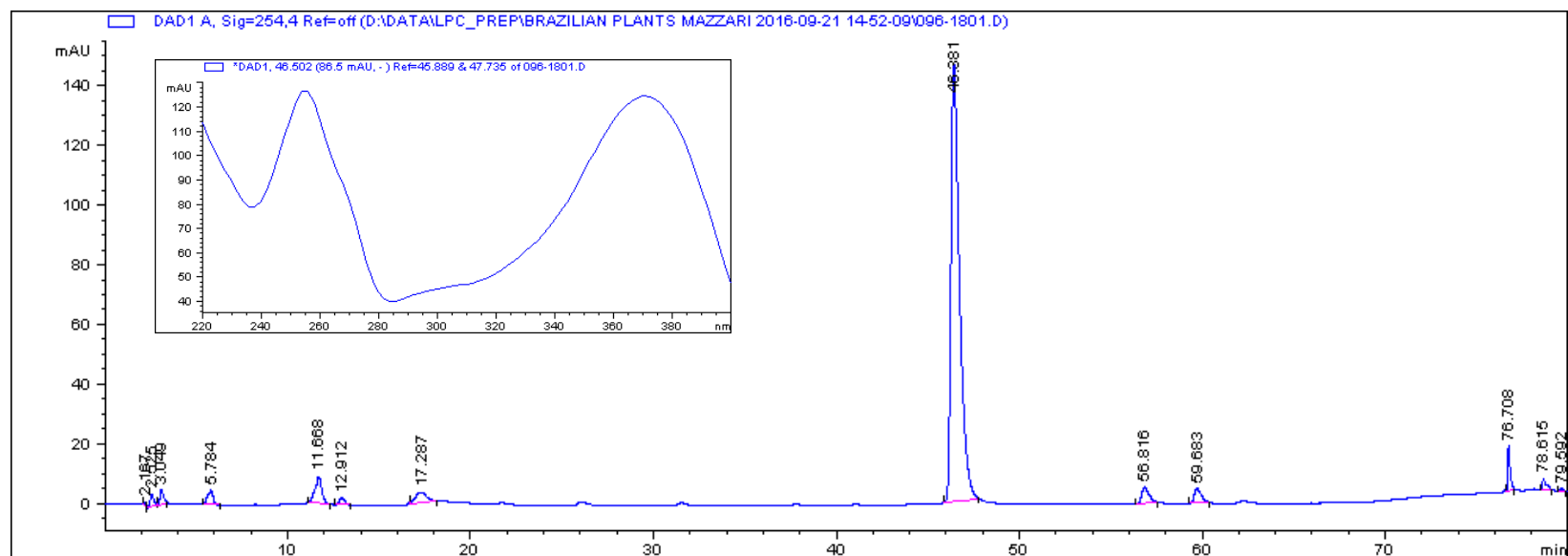
Chlorogenic acid



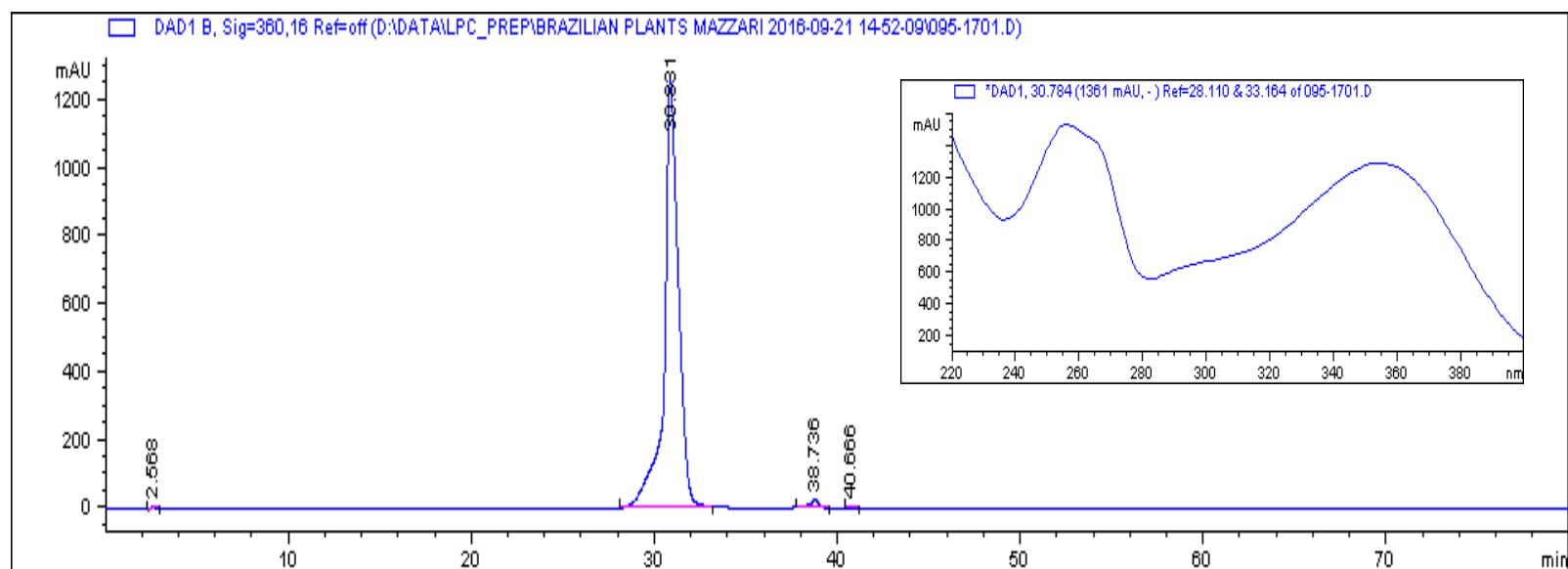
Caffeic acid



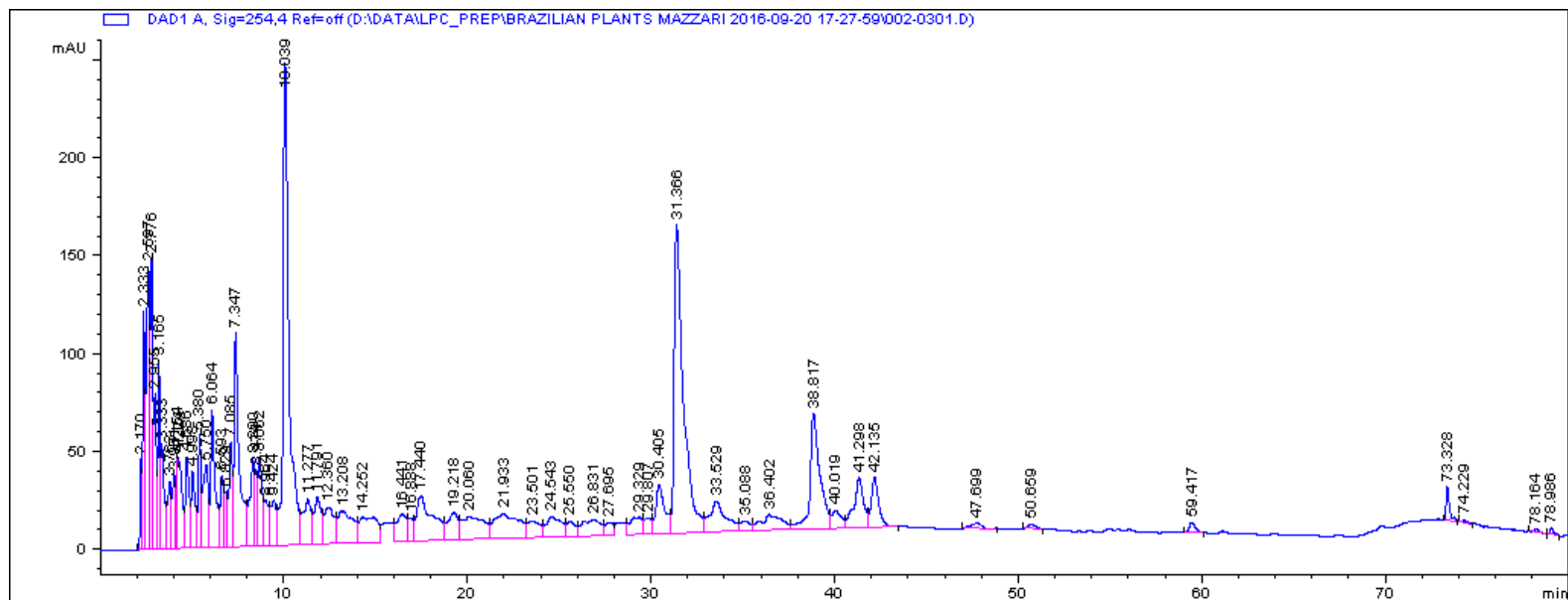
Quercetin



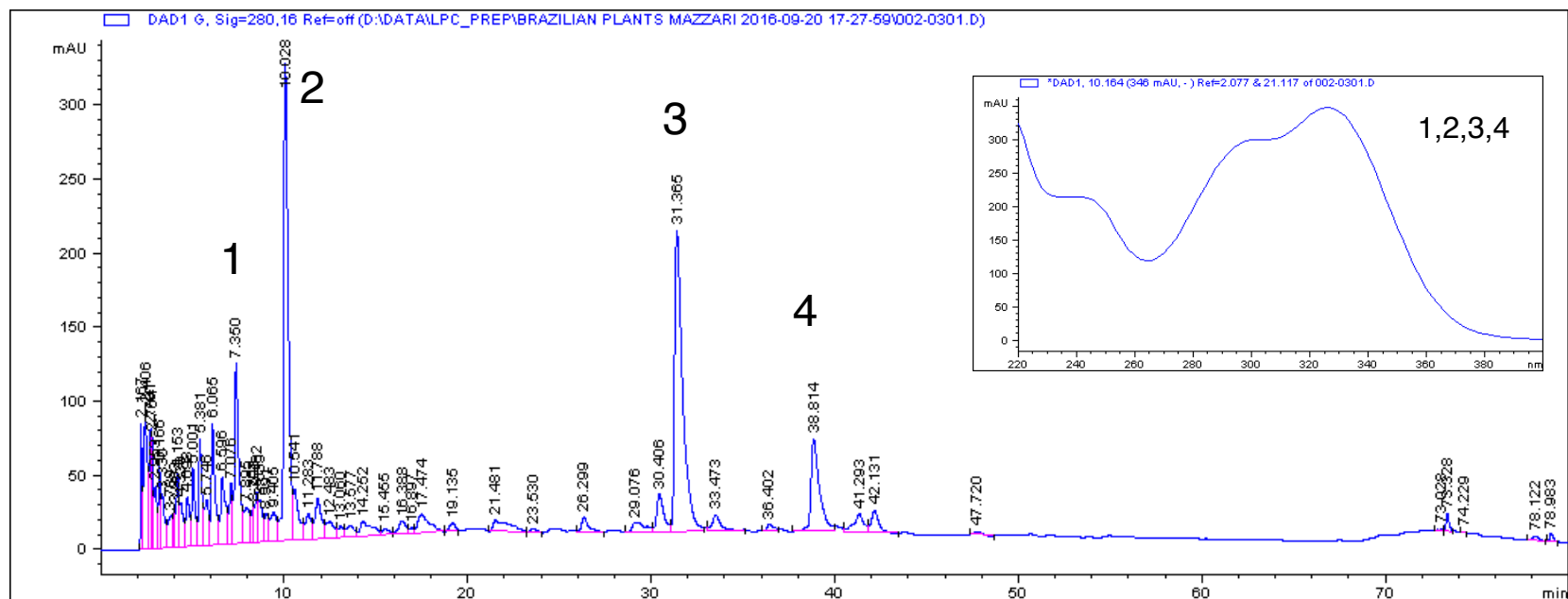
Rutin



Artemisia absinthium 254nm



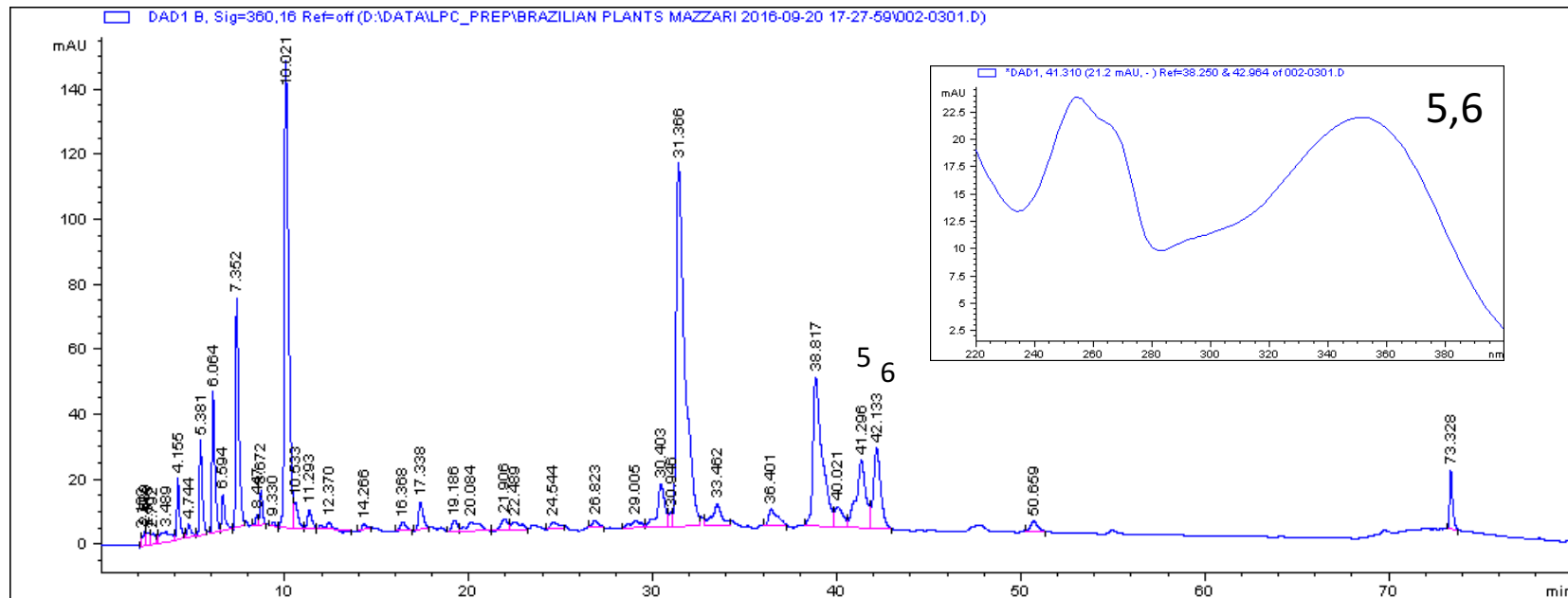
Artemisia absinthium 280nm



1,3,4 Simple phenolic acids

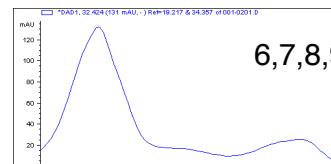
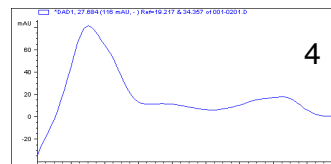
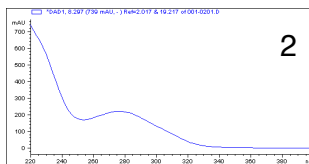
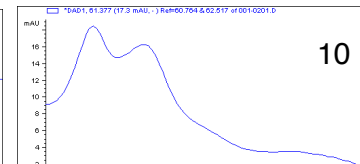
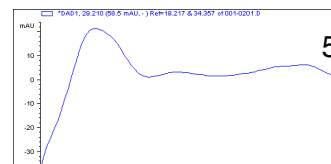
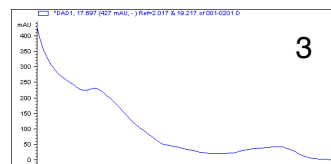
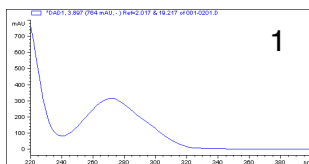
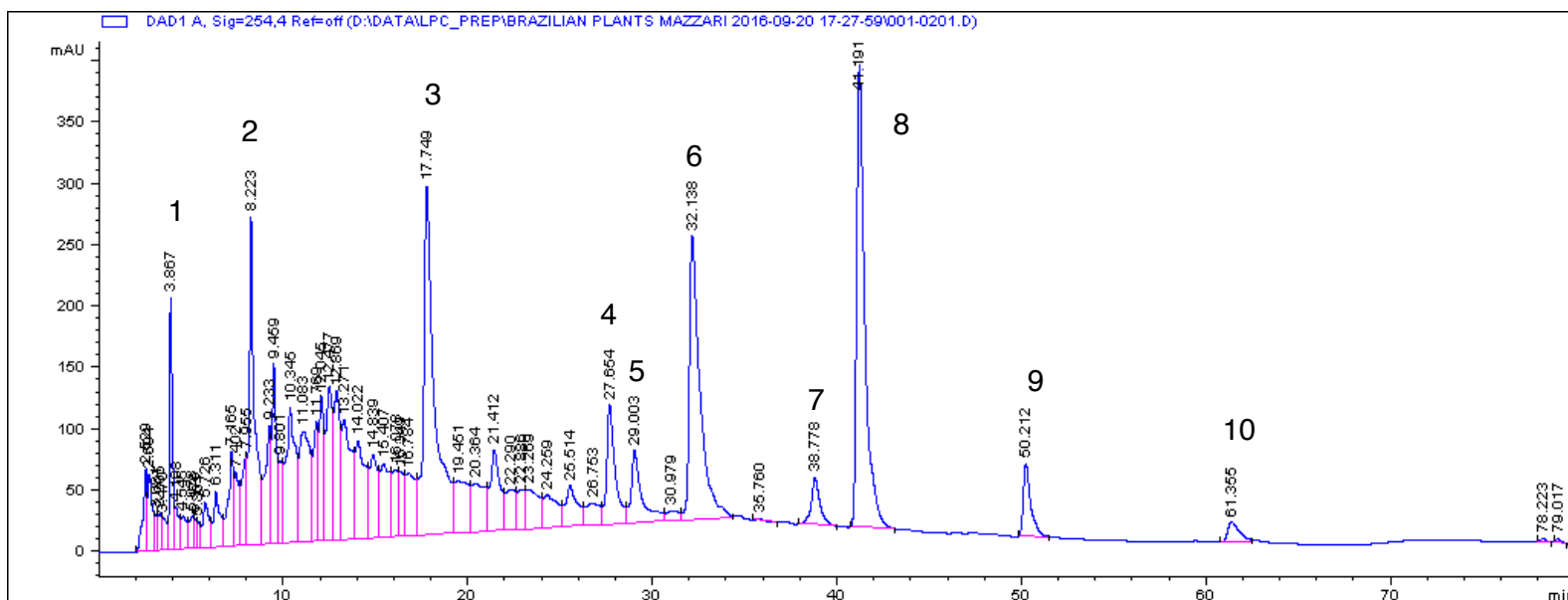
2 Chlorogenic acid

Artemisia absinthium 360nm



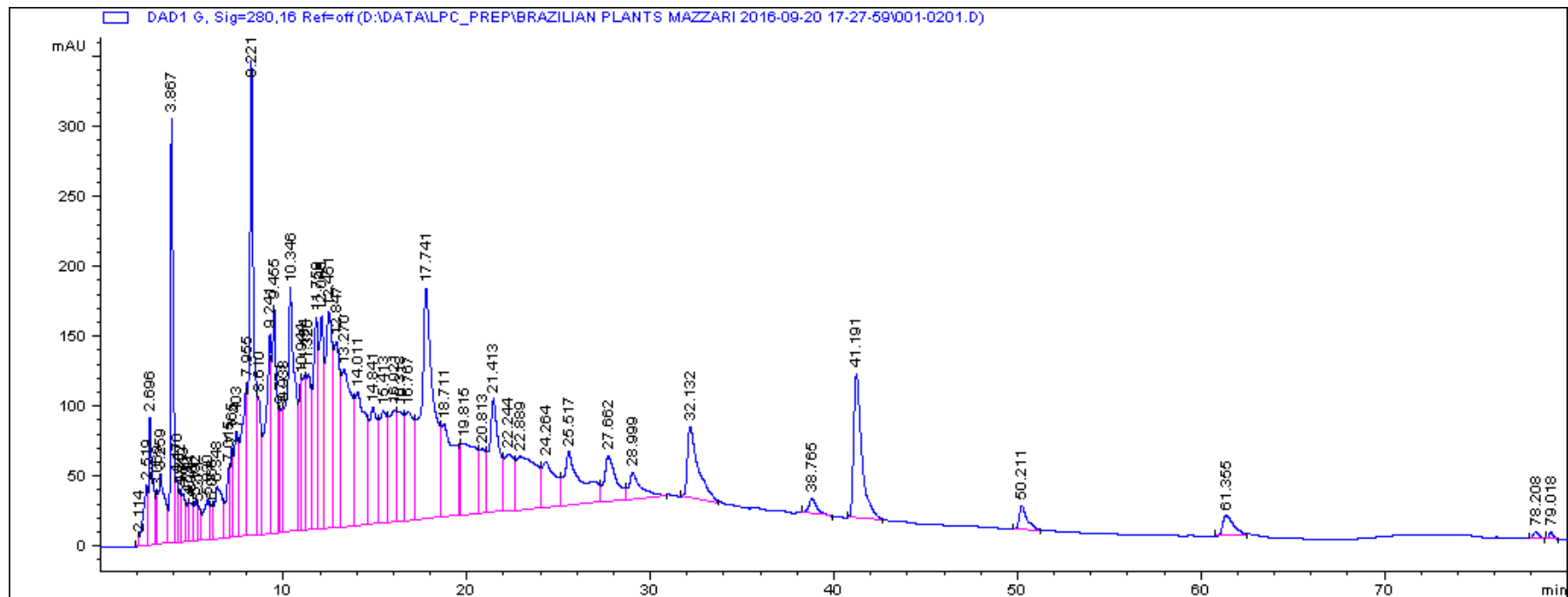
5,6 flavonoids

Apuleia ferrea 254nm

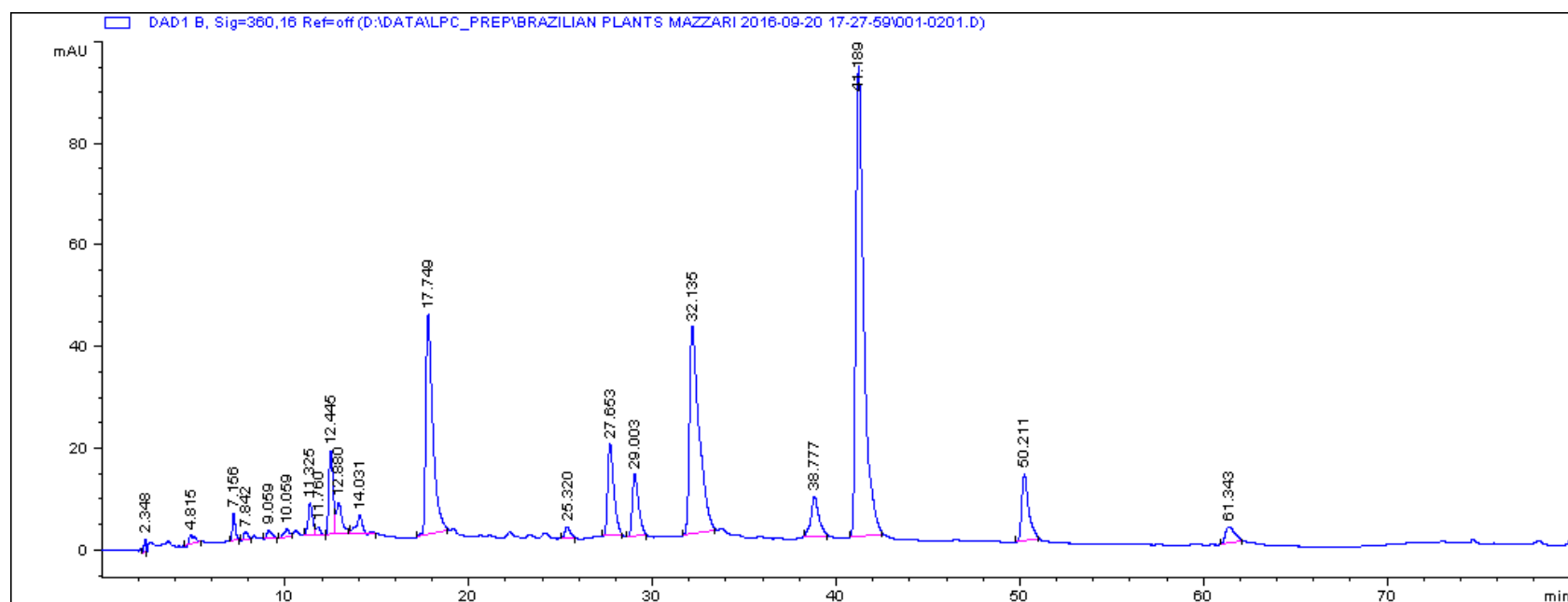


1 – 10 Not identified

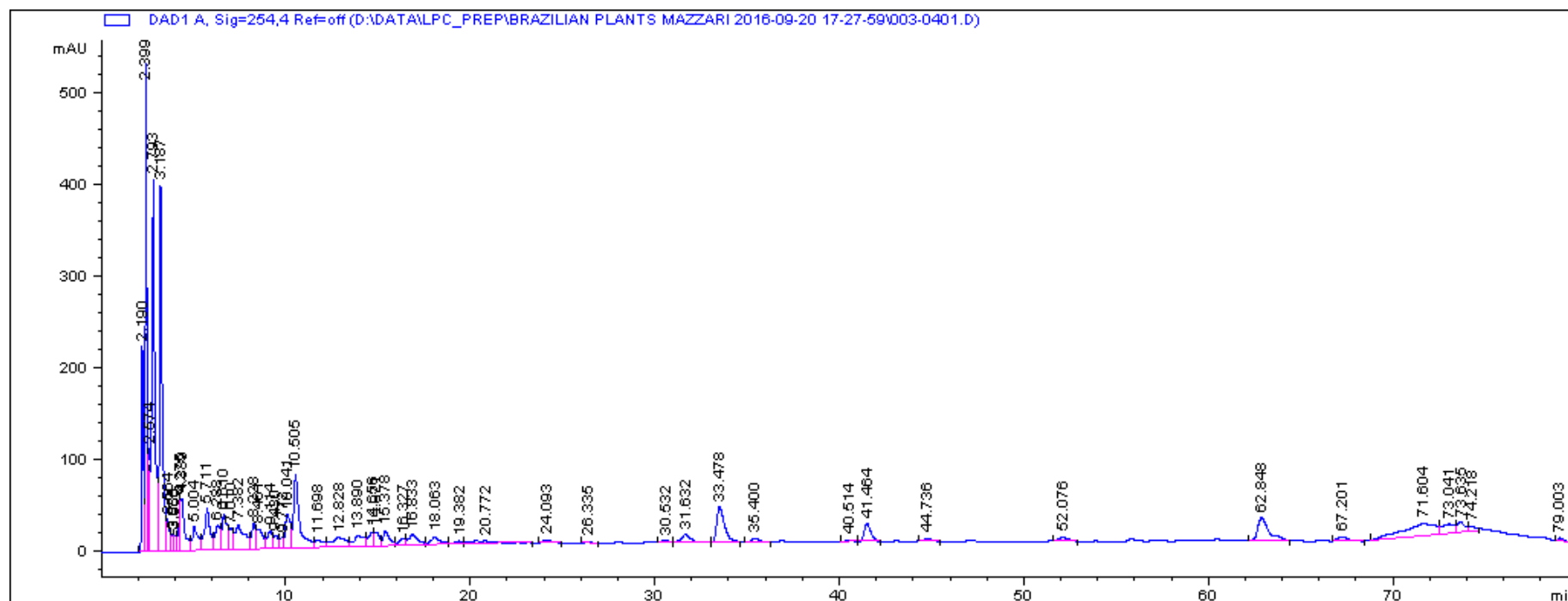
Apuleia ferrea 280nm



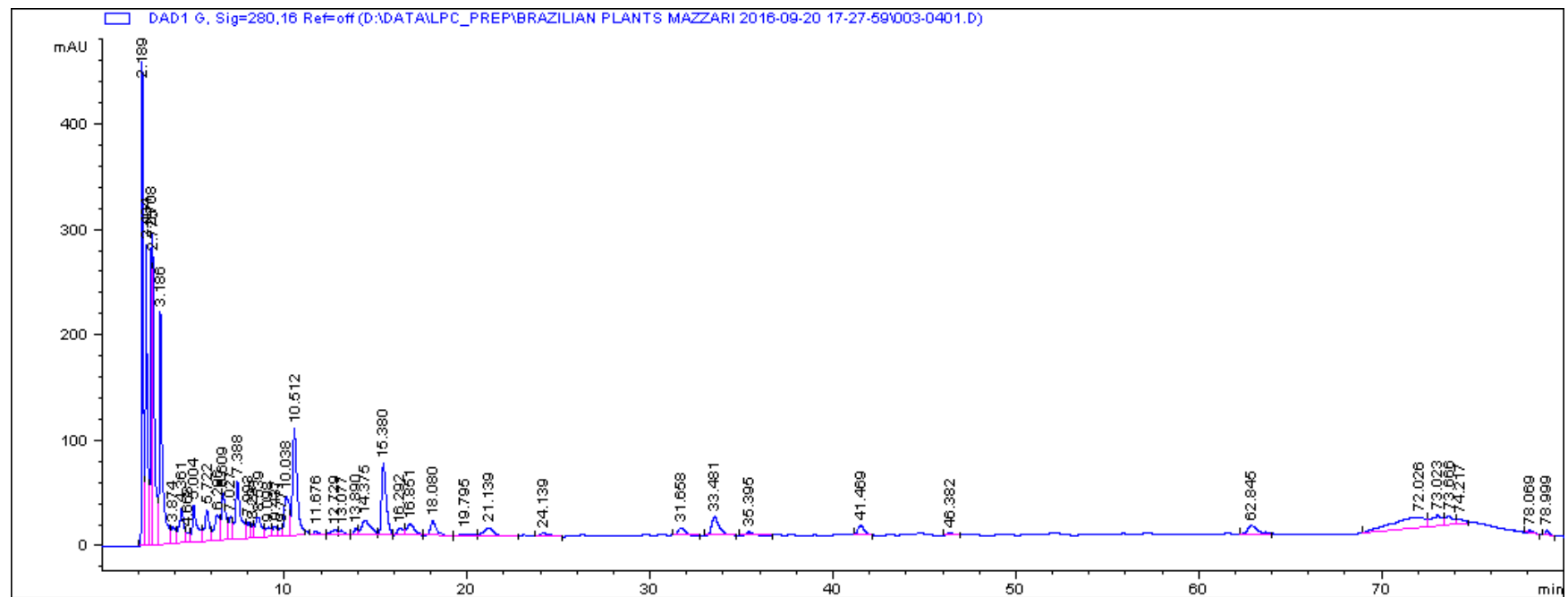
Apuleia ferrea 360nm



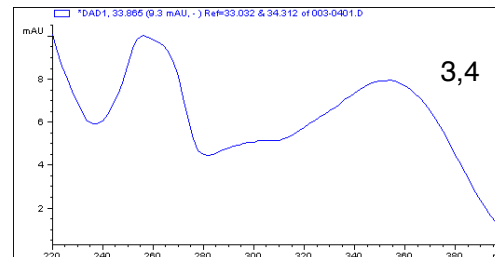
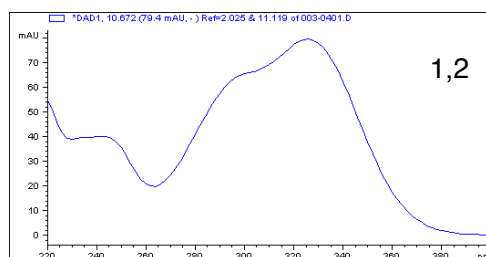
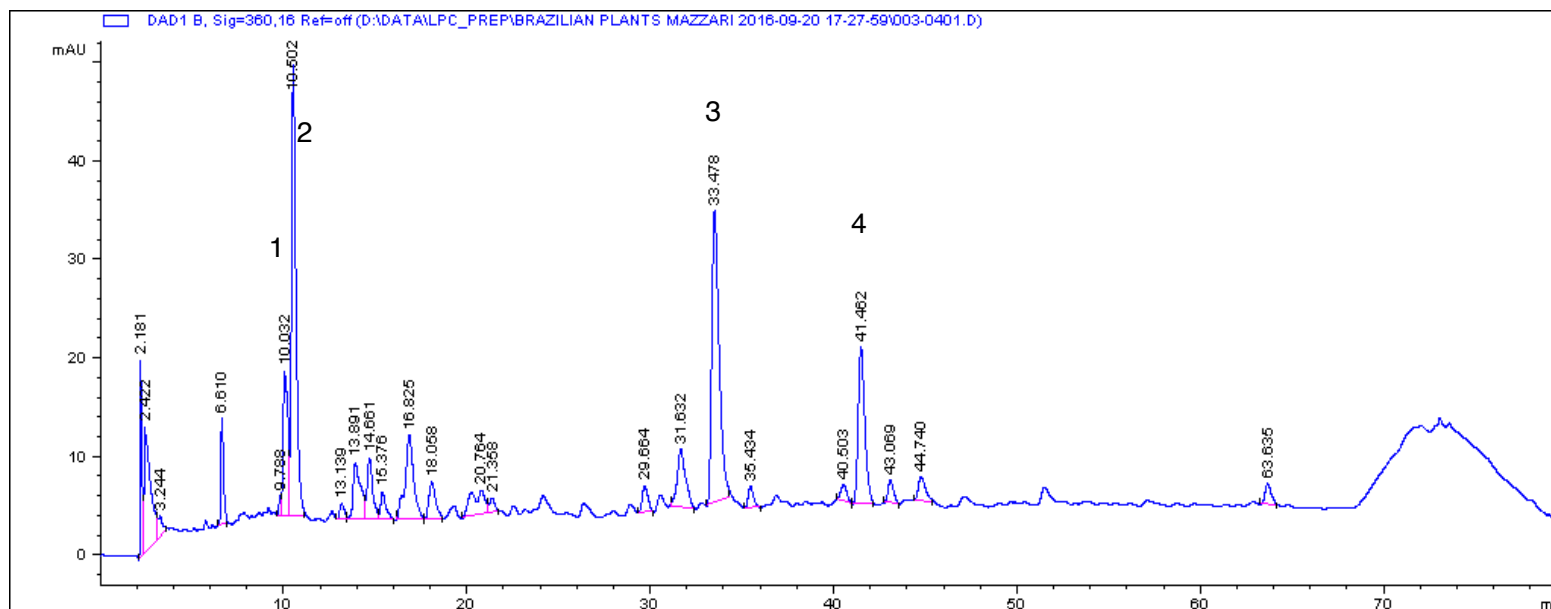
Bidens pilosa 254nm



Bidens pilosa 280nm



Bidens pilosa 360nm

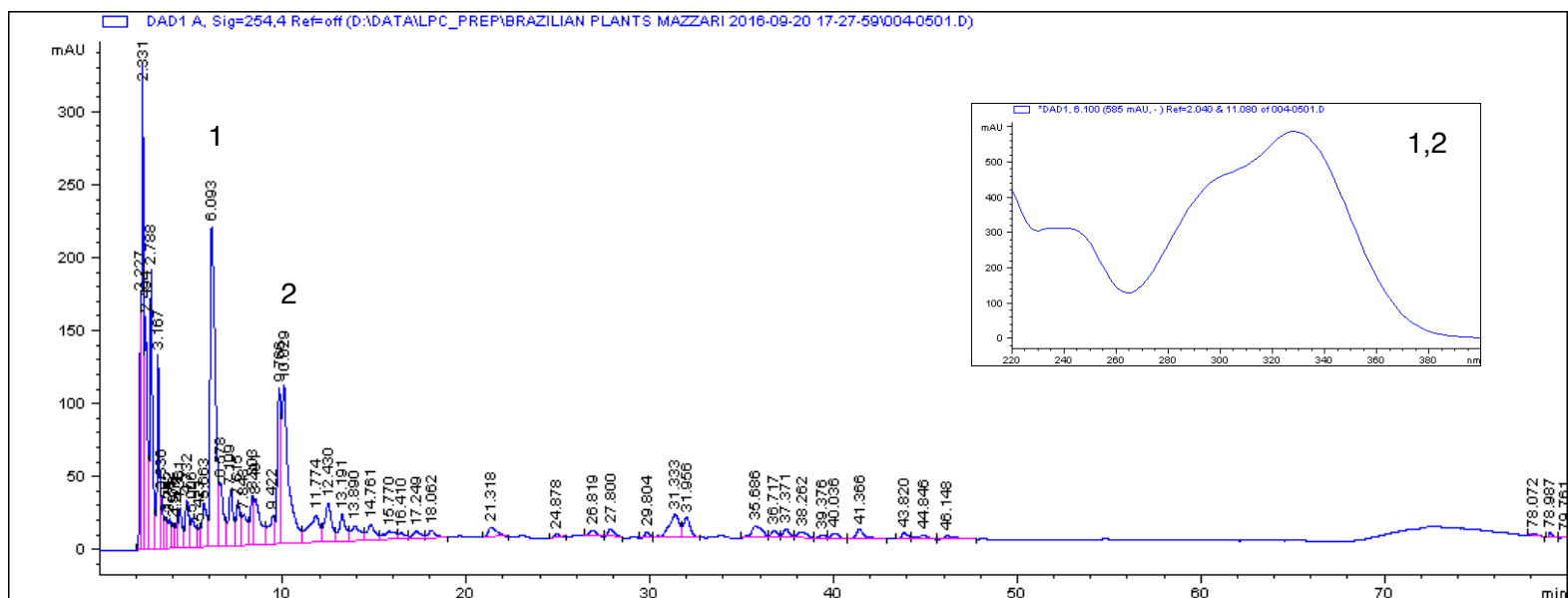


1 Chlorogenic acid

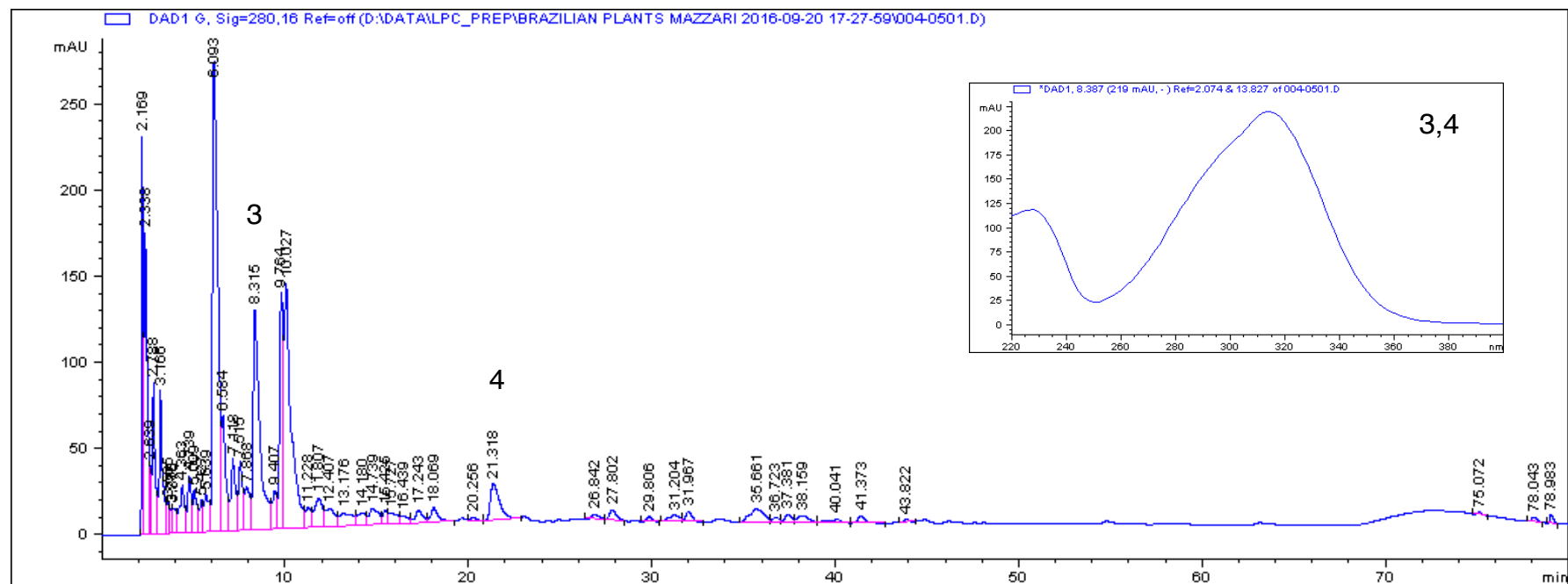
2 Caffeic acid

3,4 flavonoids

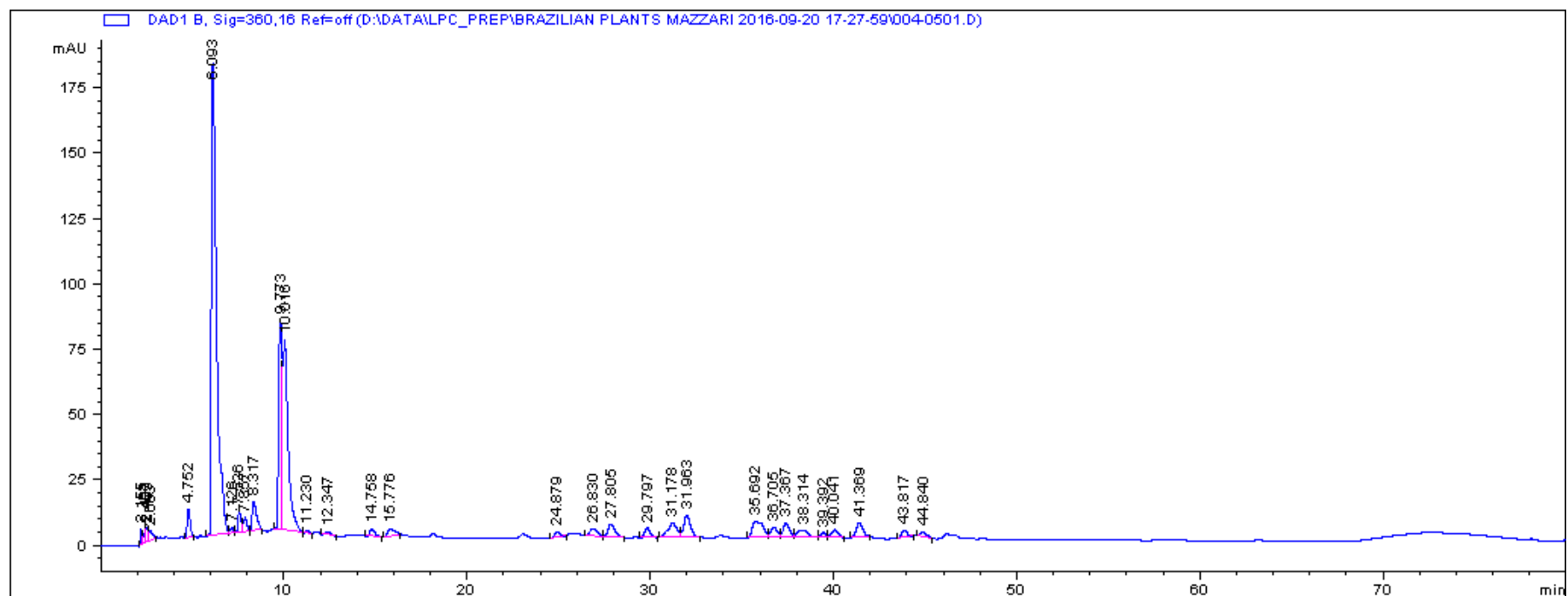
Casearia sylvestris 254nm



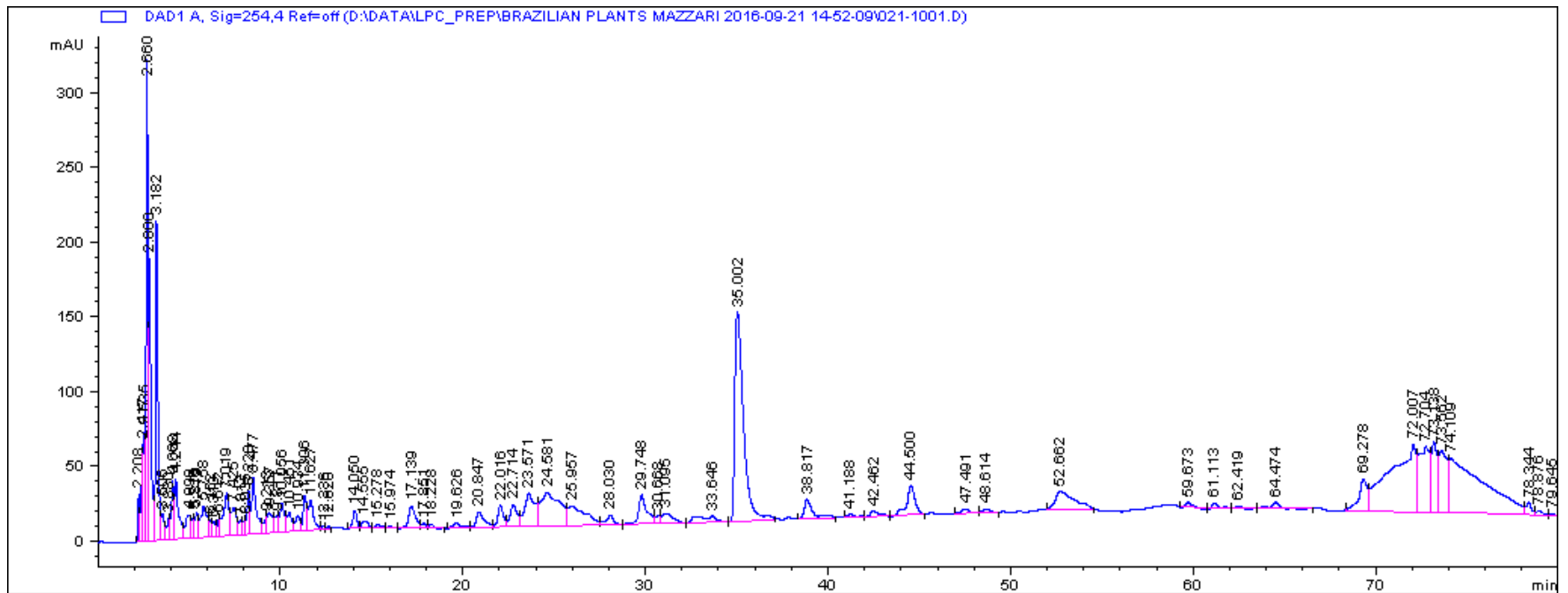
Casearia sylvestris 280nm



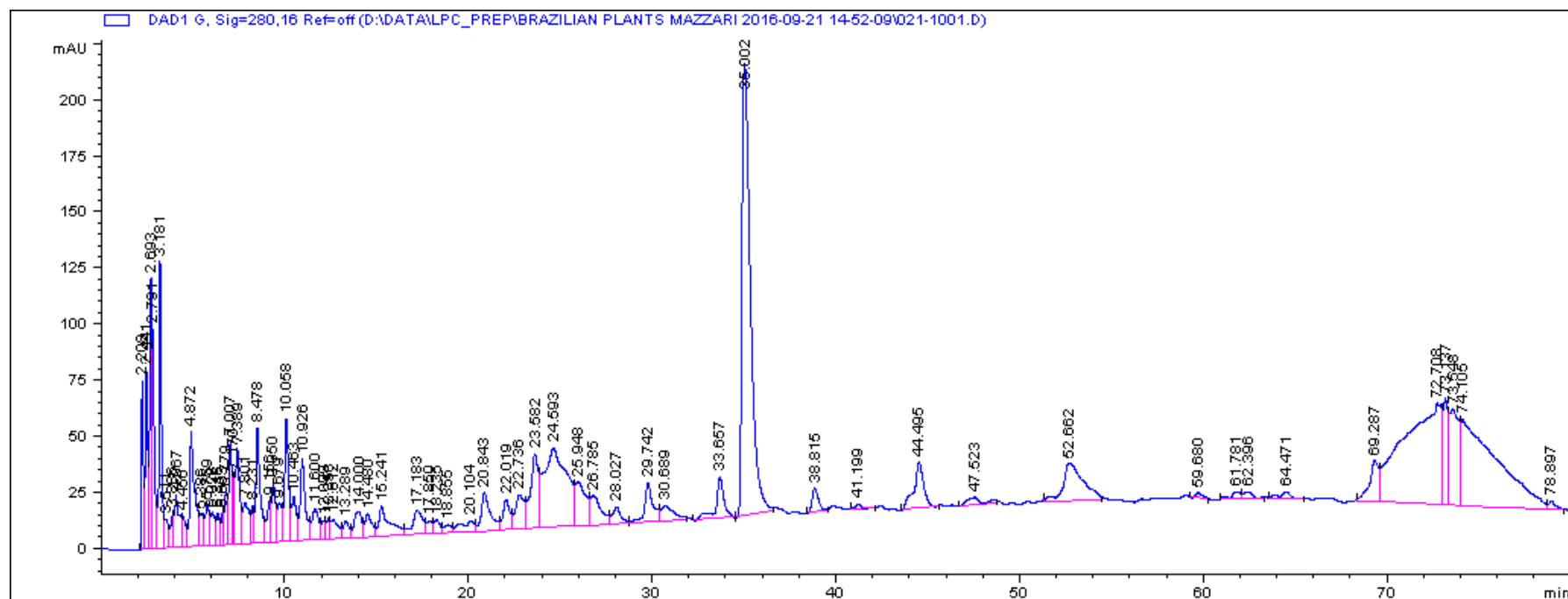
Casearia sylvestris 360nm



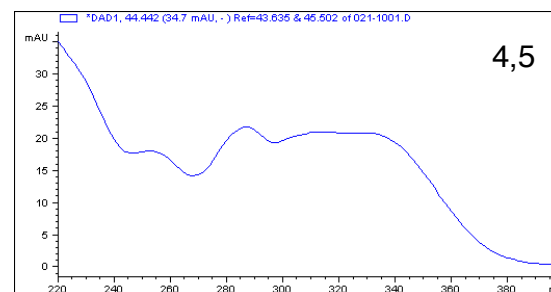
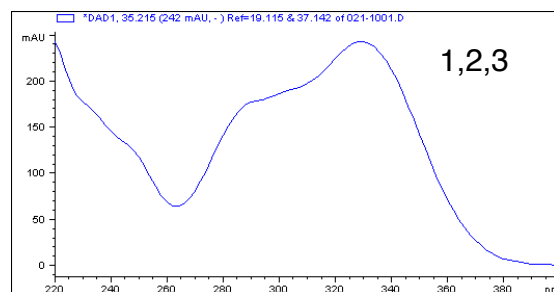
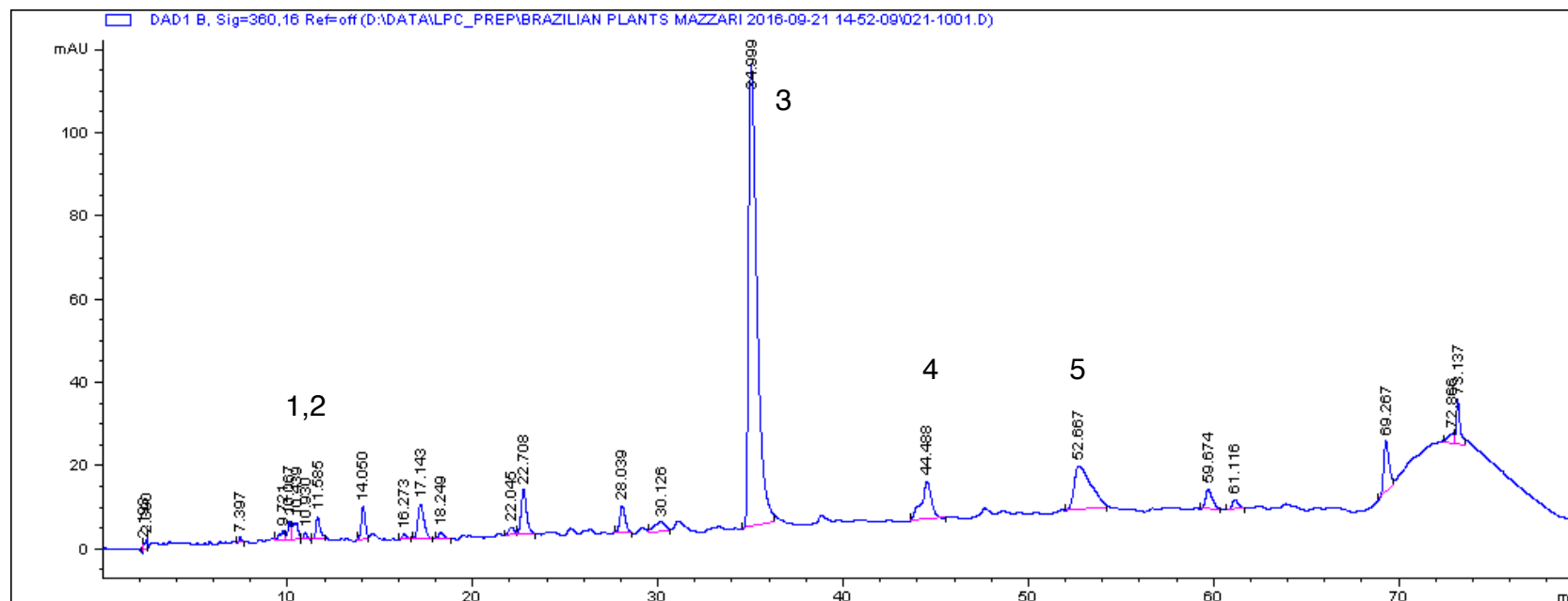
Cordia verbenaceae 254nm



Cordia verbenaceae 280nm



Cordia verbenaceae 360nm



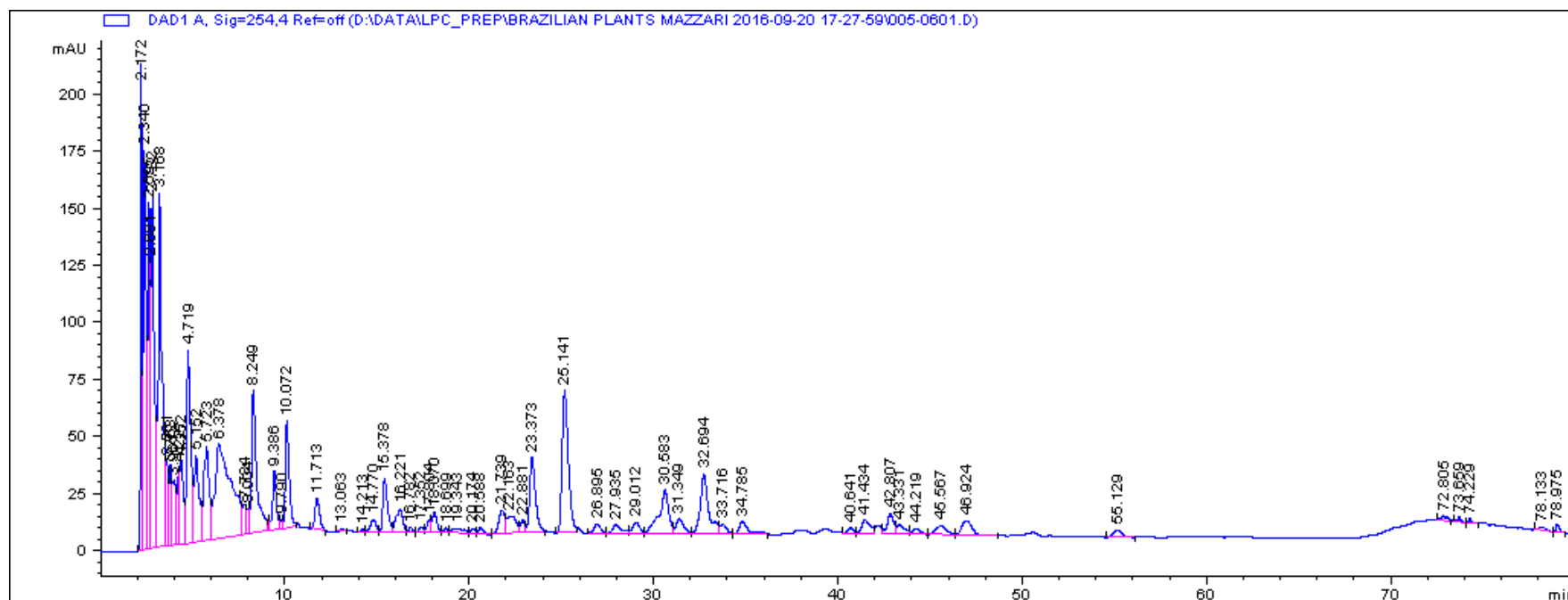
1 Chlorogenic acid

2 Caffeic acid

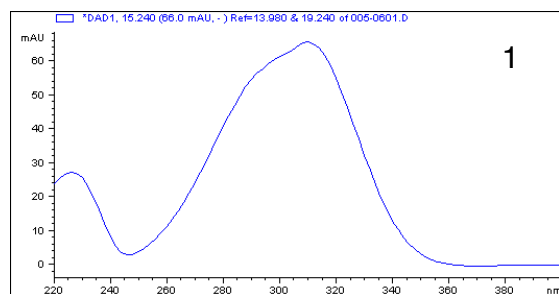
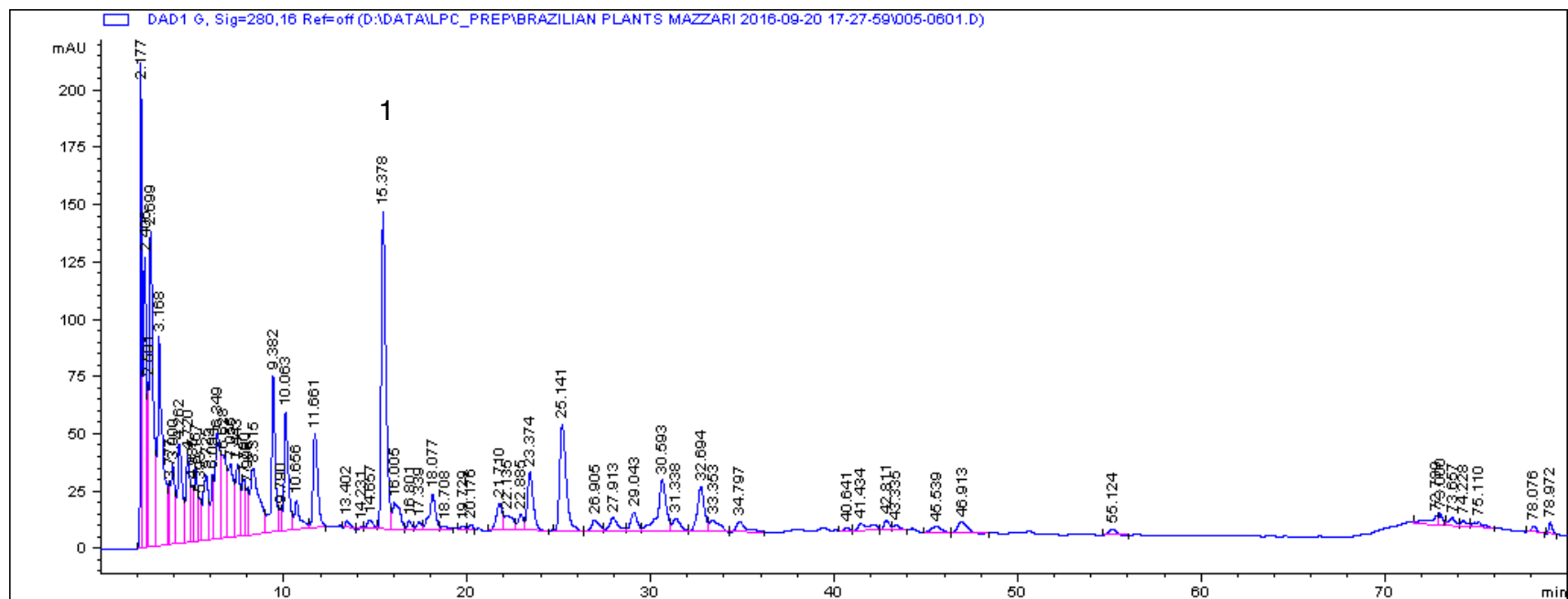
3 Phenolic acid

4, 5 Not identified

Costus spicatus 254nm

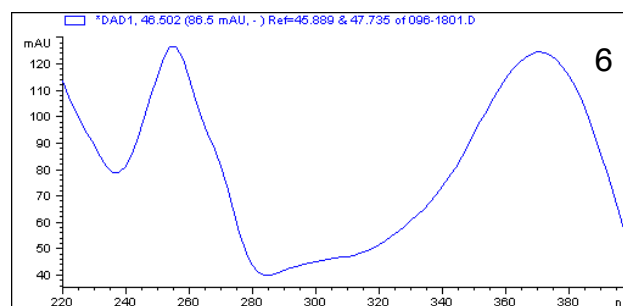
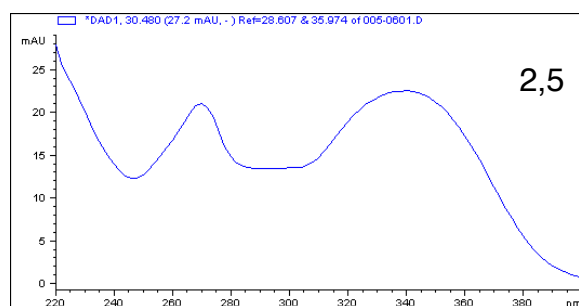
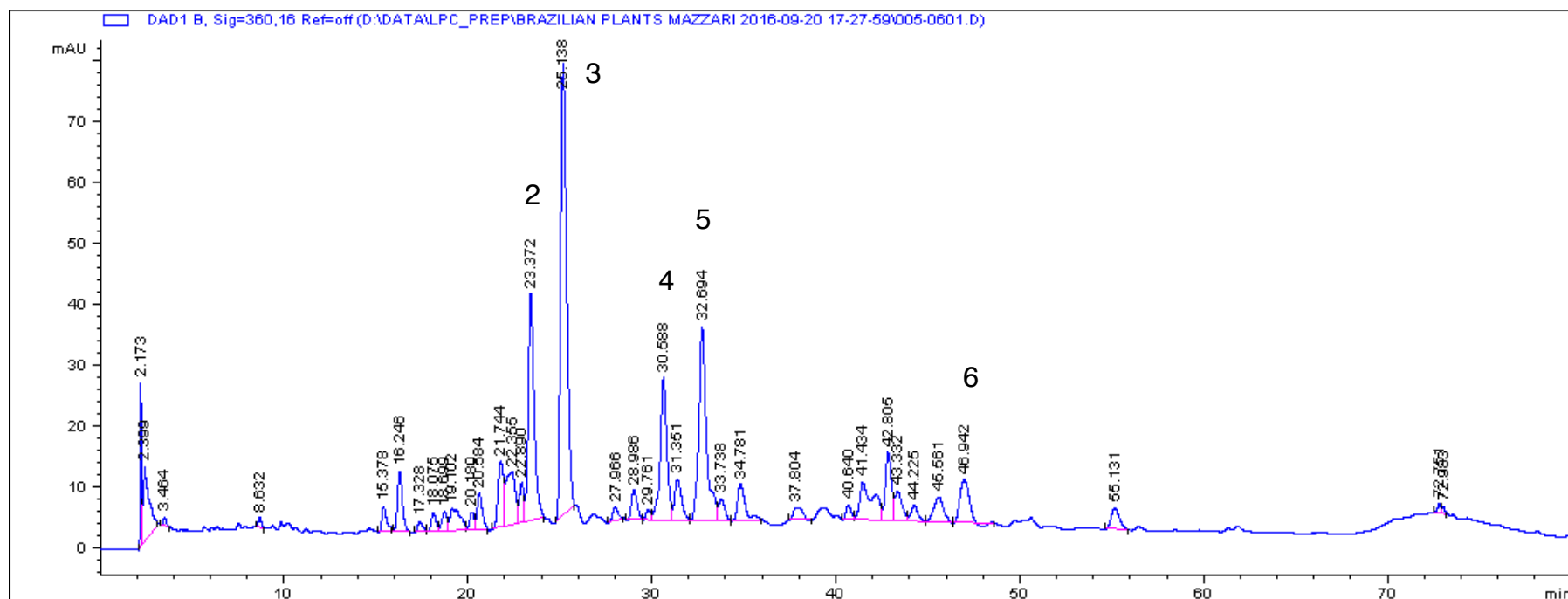


Costus spicatus 280nm



1 Not identified

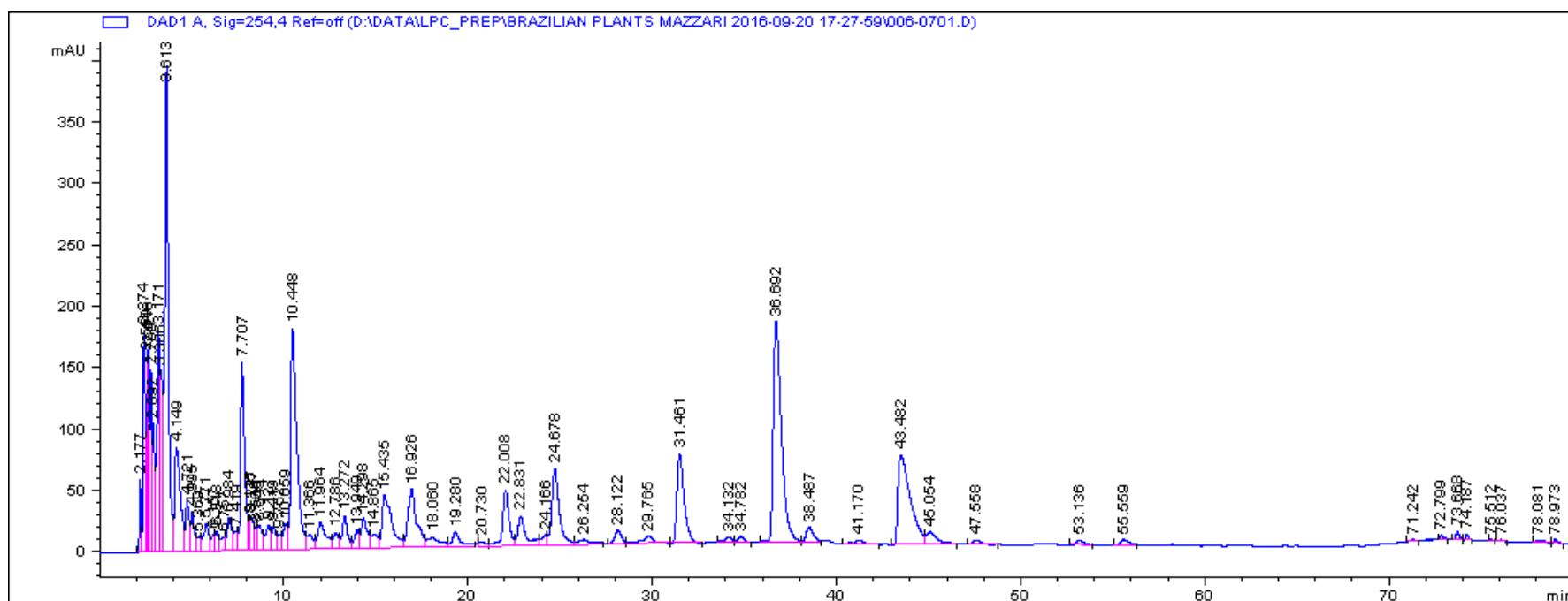
Costus spicatus 360nm



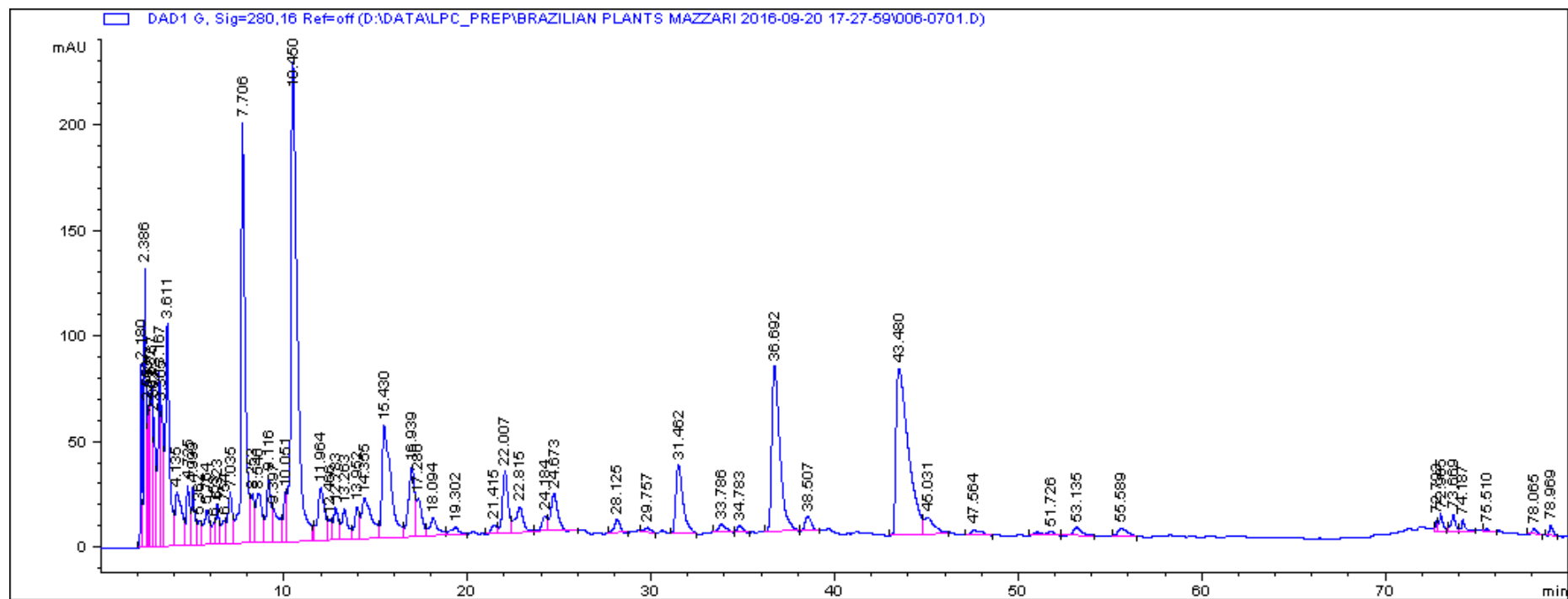
2-5 Glycosilated flavonoids

6 Quercetin

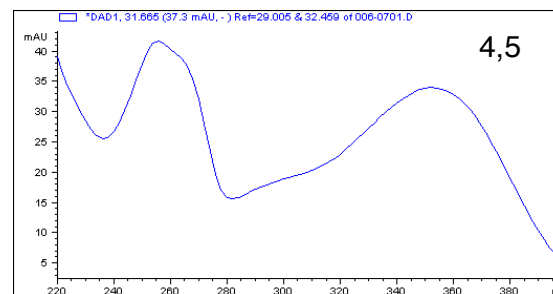
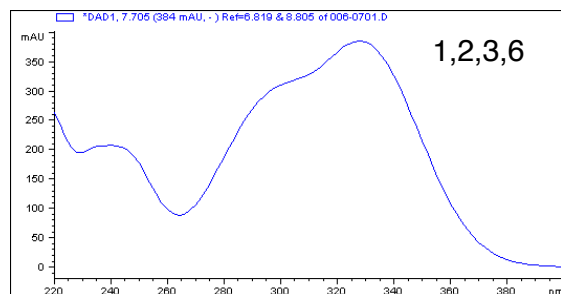
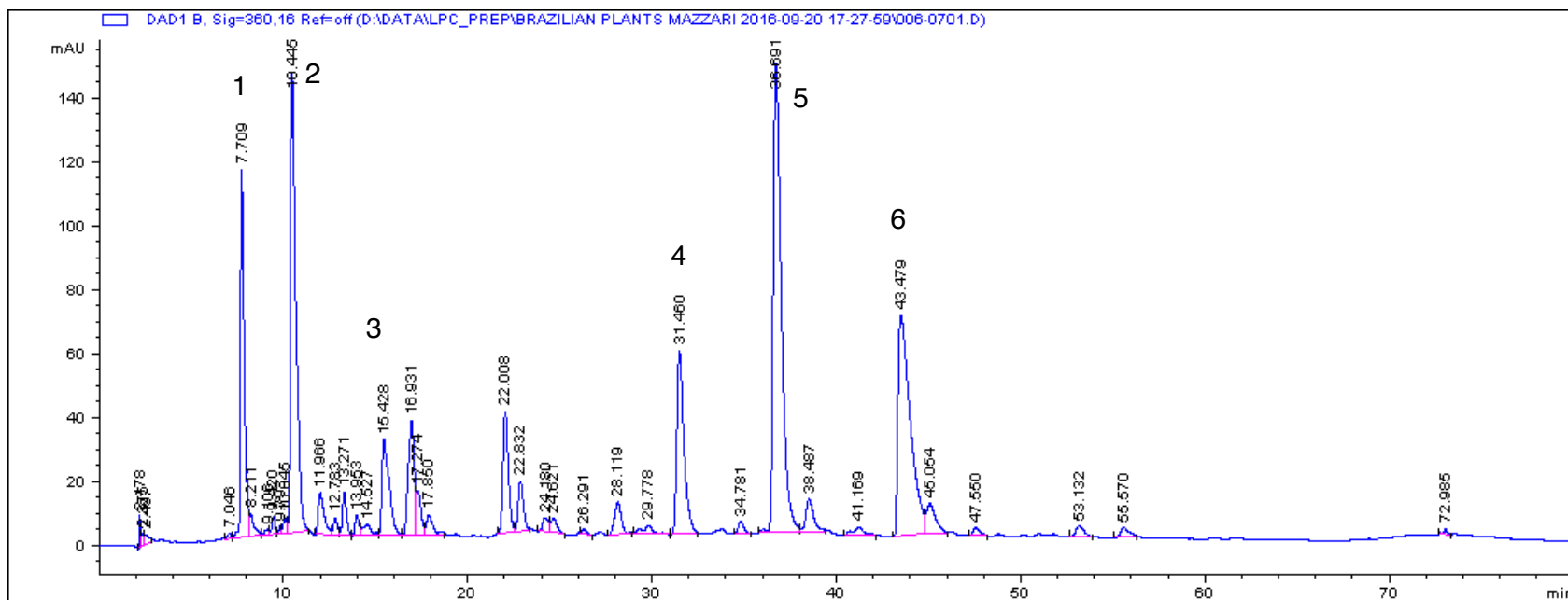
Equisetum arvense 254nm



Equisetum arvense 280nm



Equisetum arvense 360nm

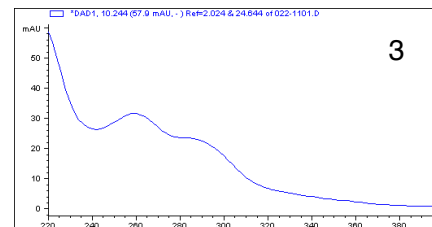
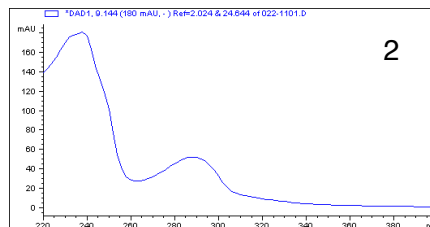
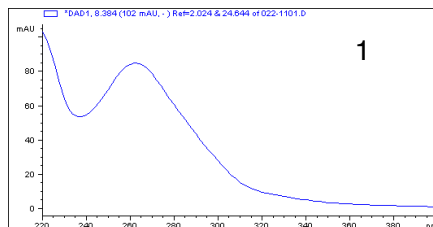
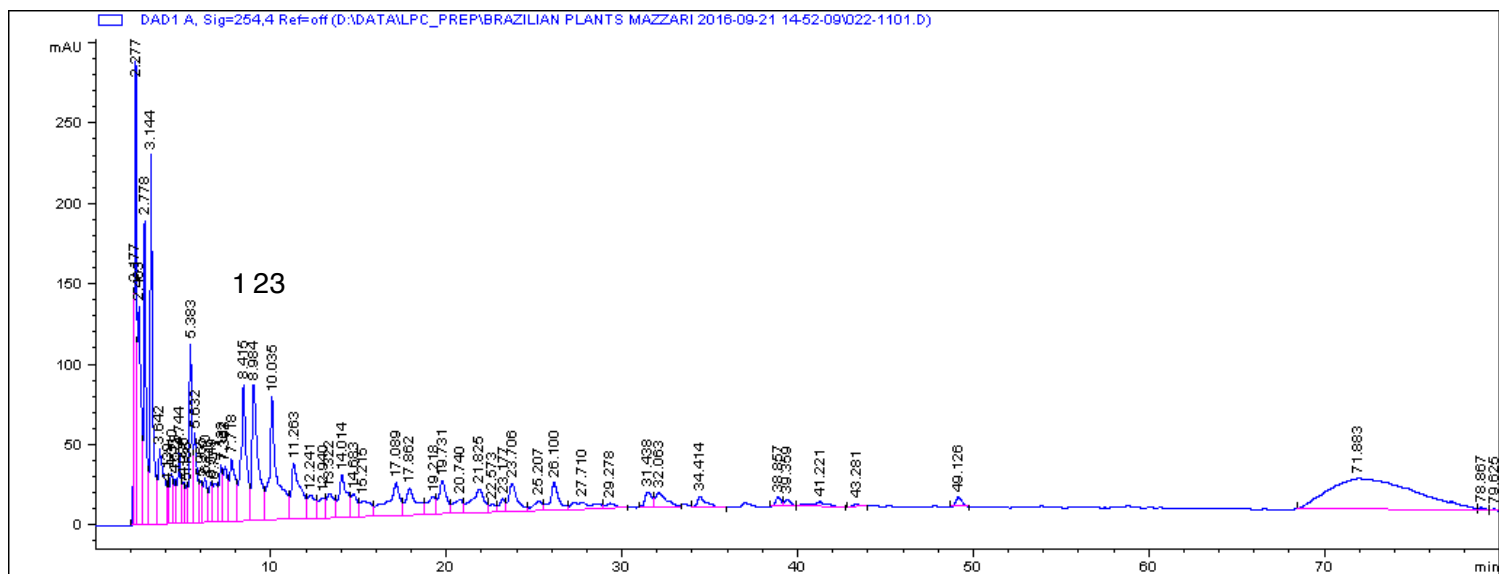


1,3,6 Phenolic acid

2 Caffeic acid

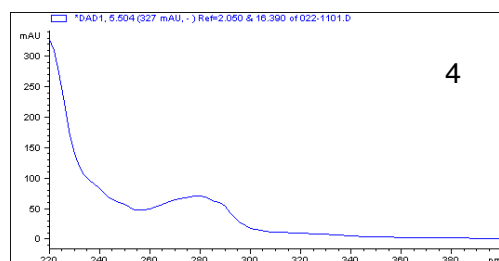
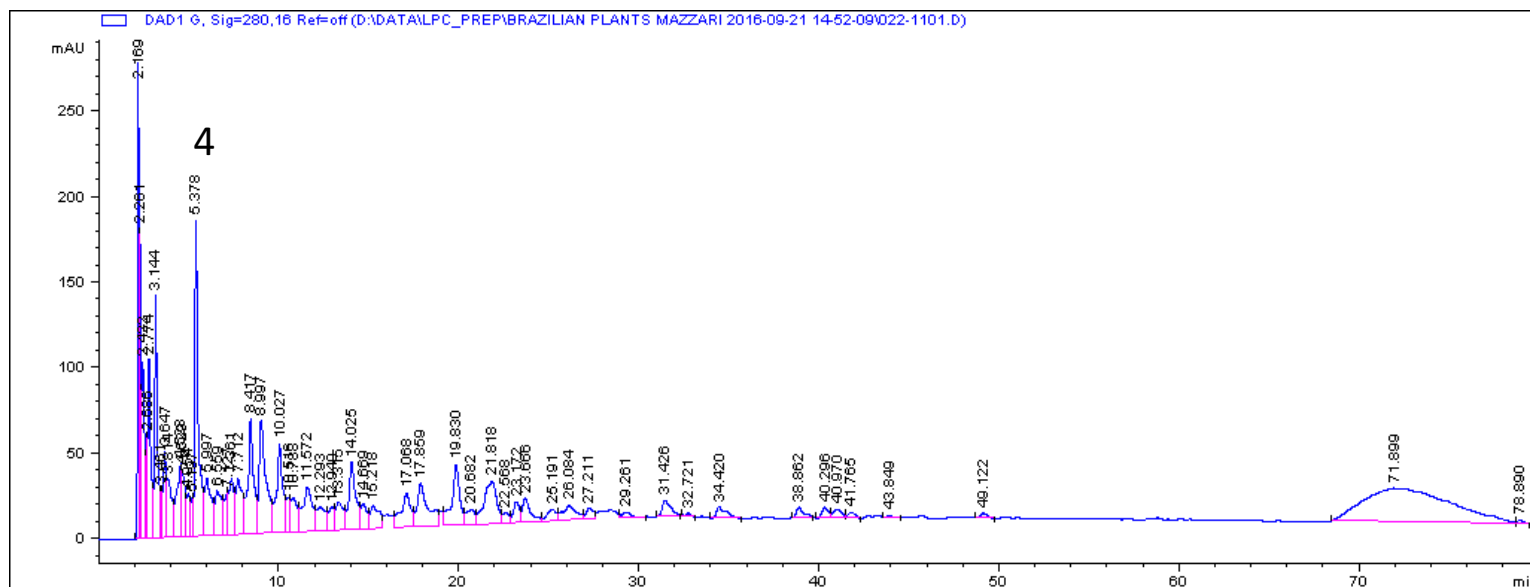
4,5 Glycosilated
flavonoids

Erythrina mulungu 254nm



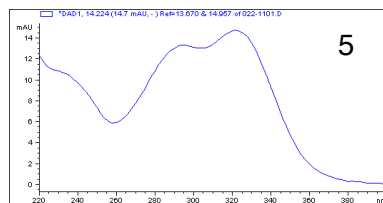
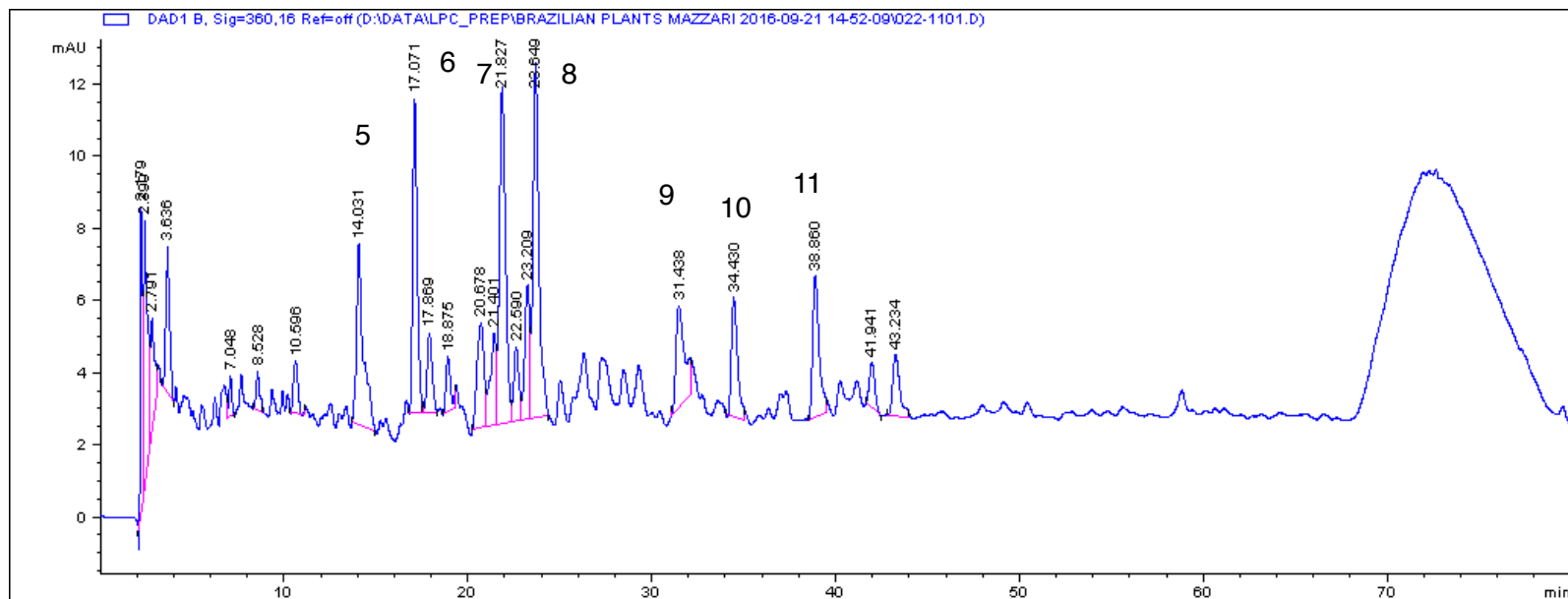
1,2,3
Tannins

Erythrina mulungu 280nm

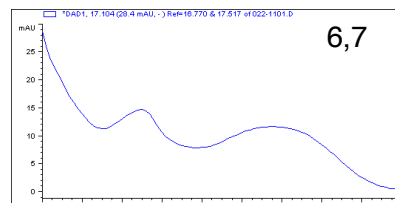


4 Tannins

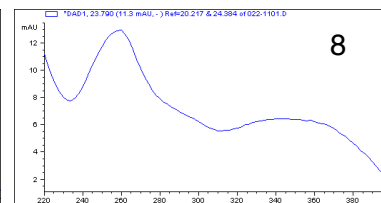
Erythrina mulungu 360nm



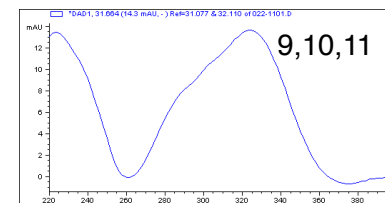
5 Phenolic acid



6,7 Glycosilated flavonoids

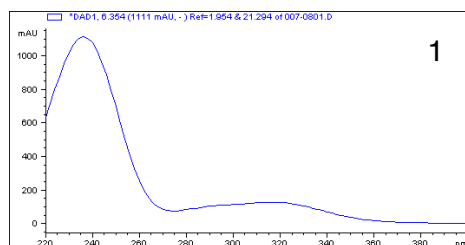
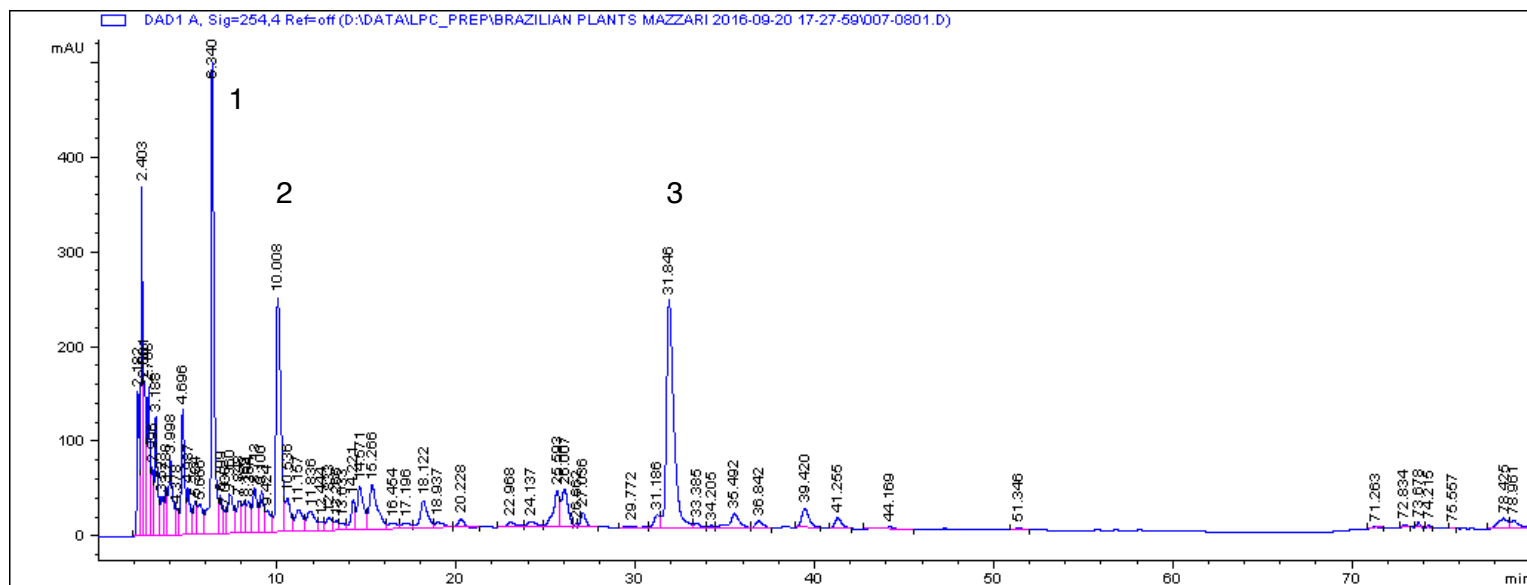


8 Glycosilated flavonoids

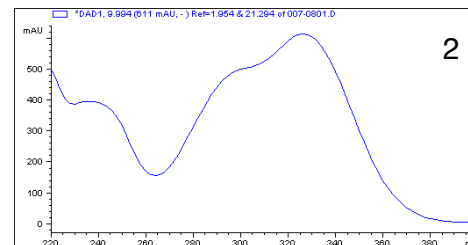


9,10,11 Phenolic acids

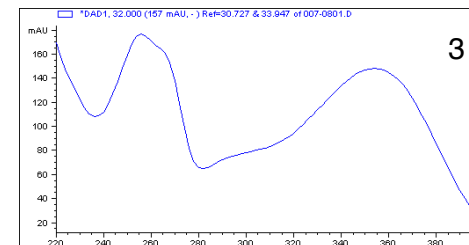
Lamium album 254nm



1 Tannin

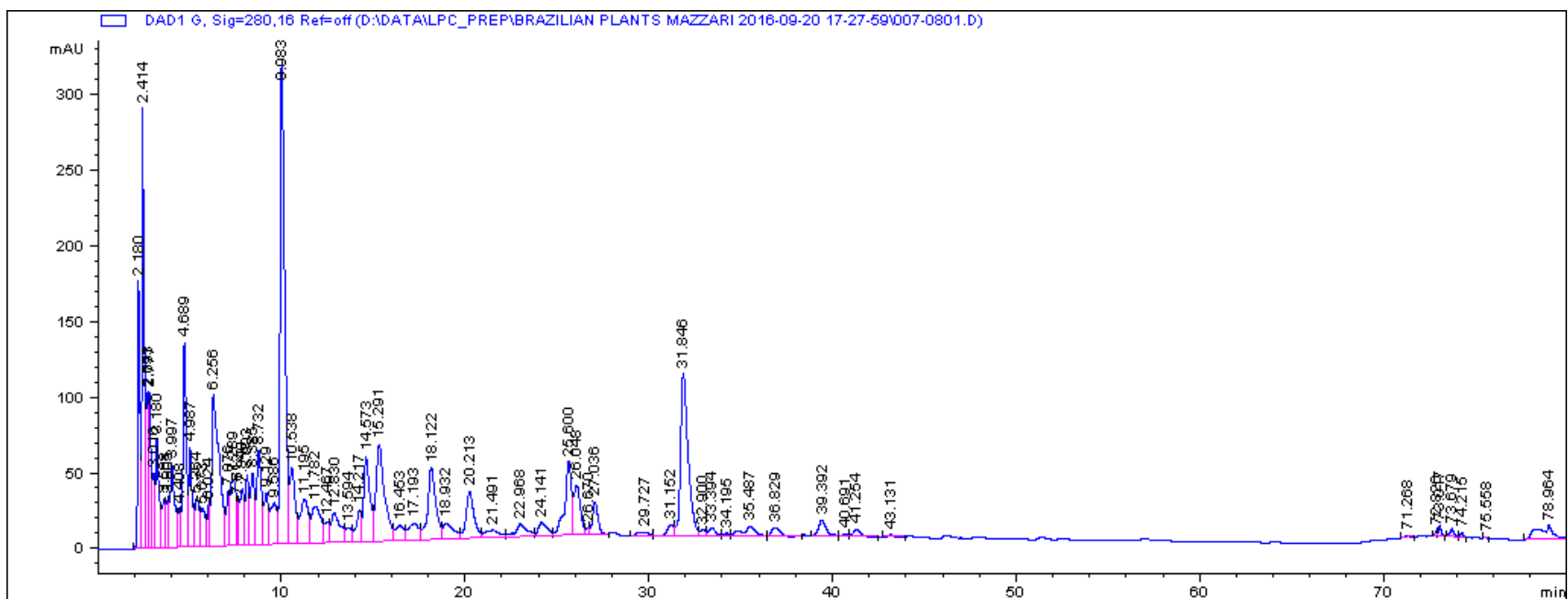


2 Chlorogenic acid

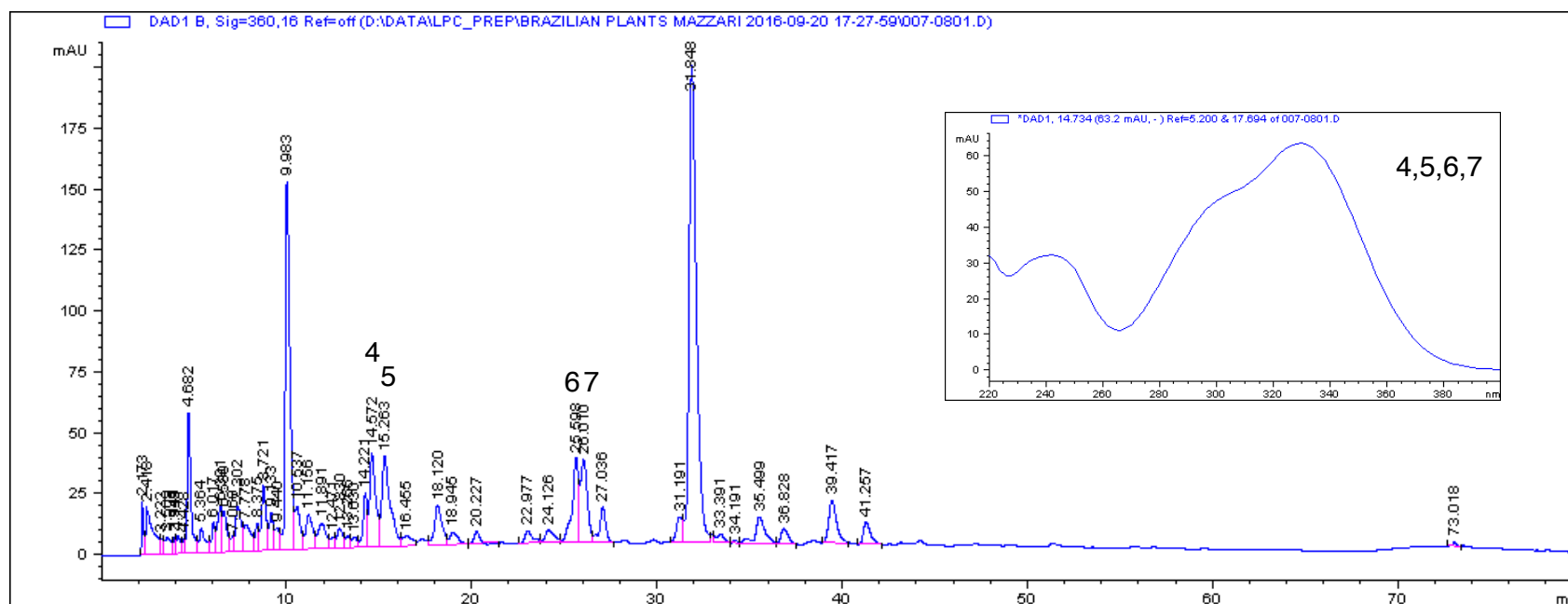


3 Glycosilated flavonoid

Lamium album 280nm

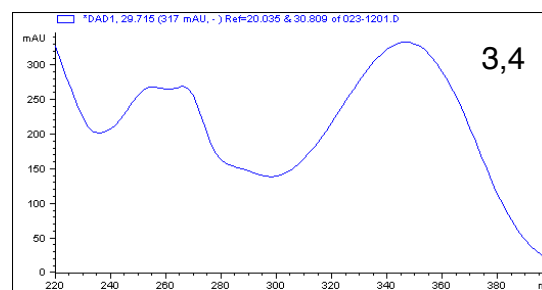
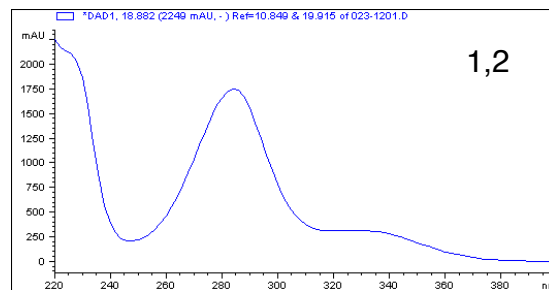
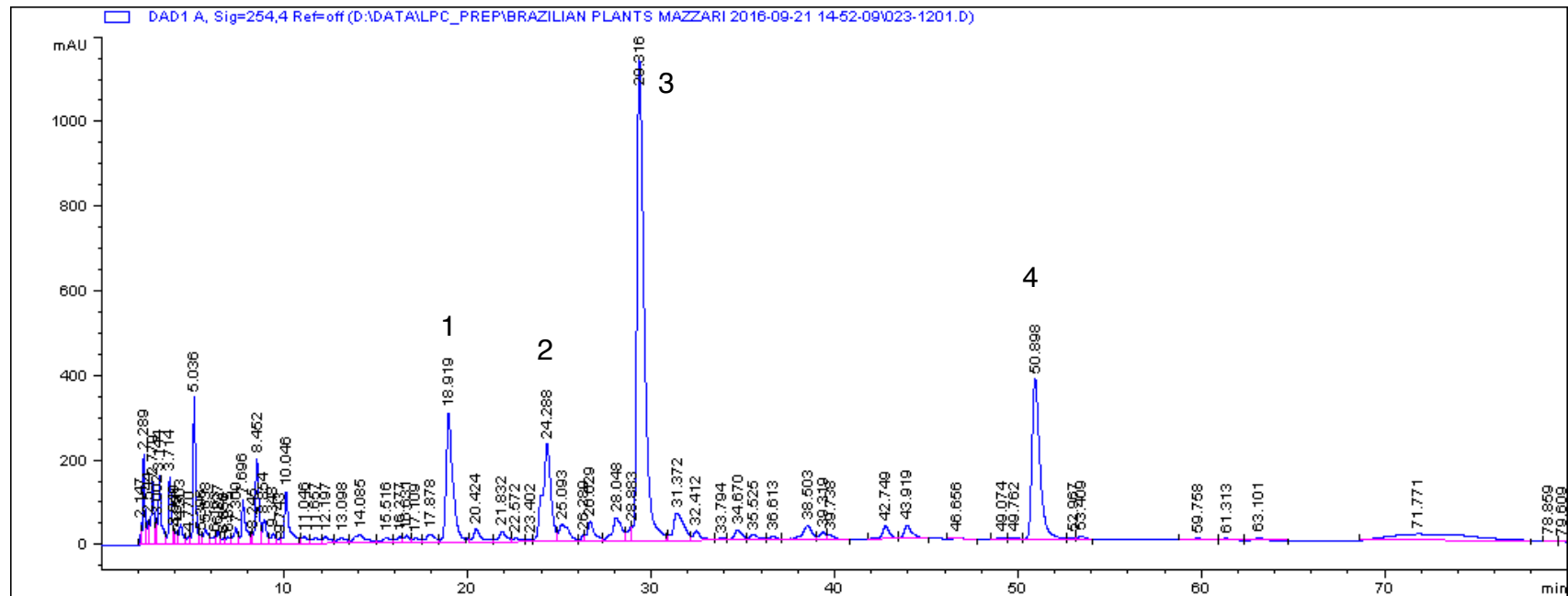


Lamium album 360nm



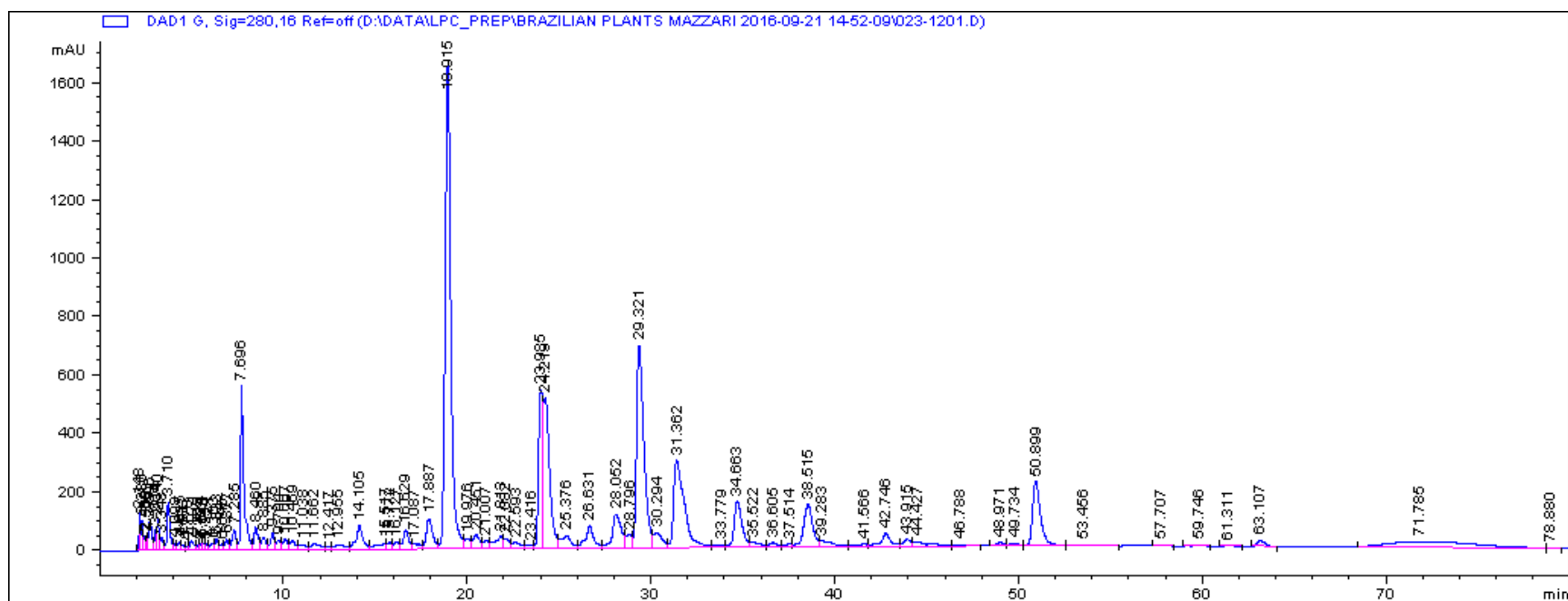
4-7 Phenolic acids

Lippia sidoides 254nm

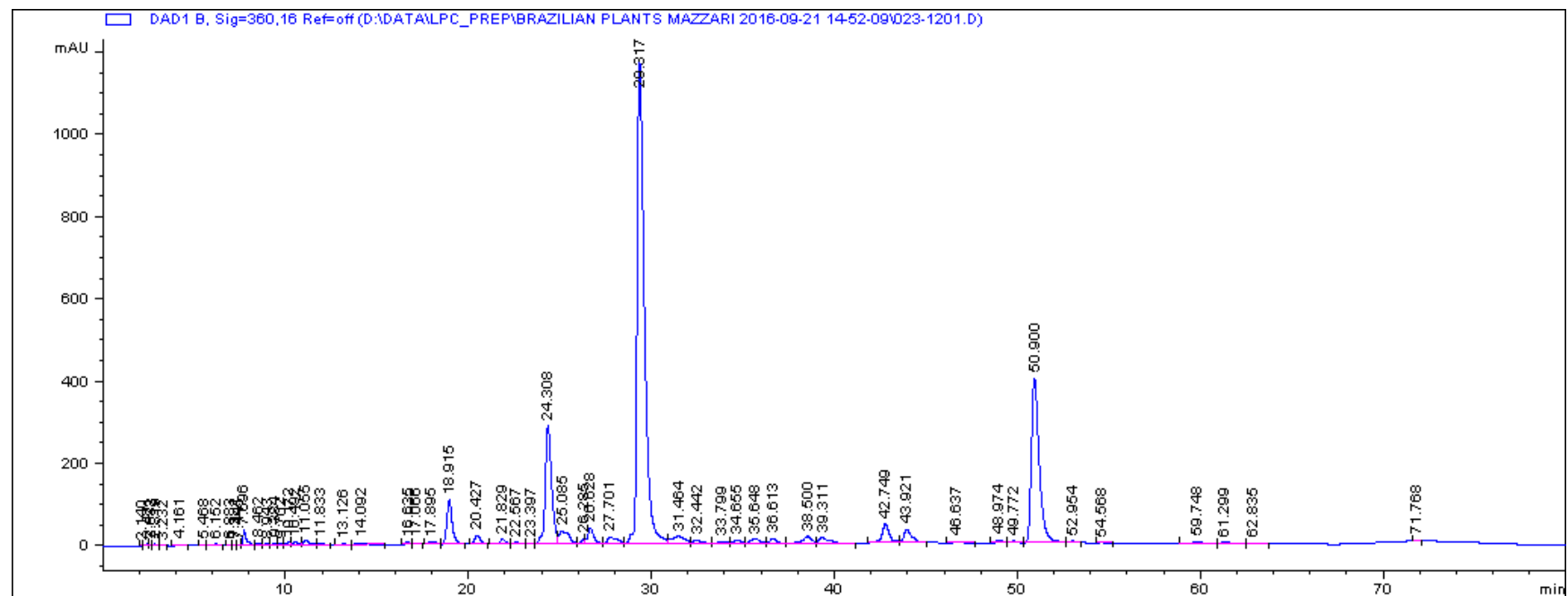


3,4 Flavonoids
1,2 Not identified

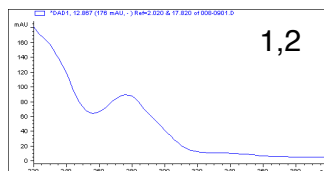
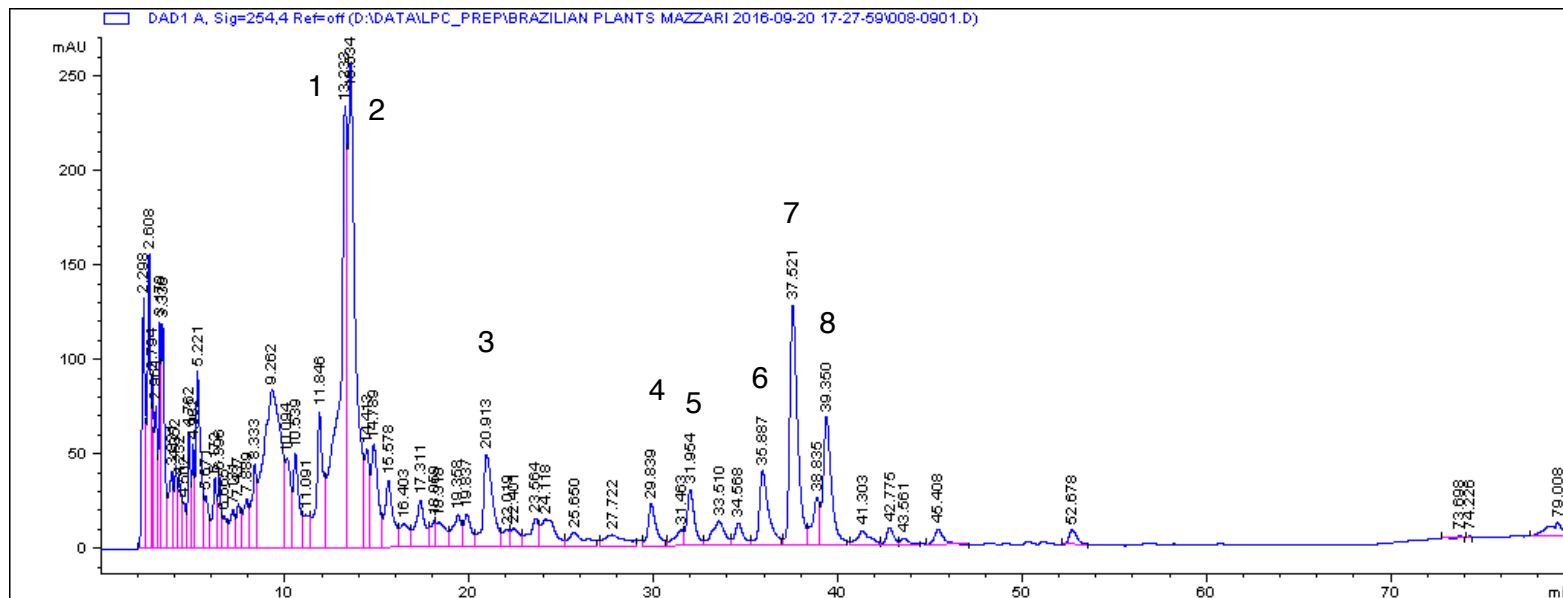
Lippia sidoides 280nm



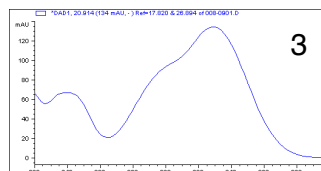
Lippia sidoides 360nm



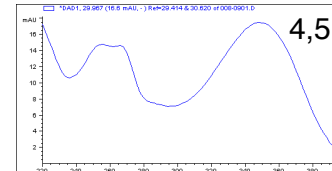
Malva sylvestris (flower) 254nm



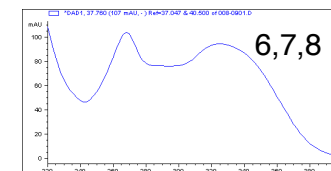
1,2 Not identified



3 Phenolic acid

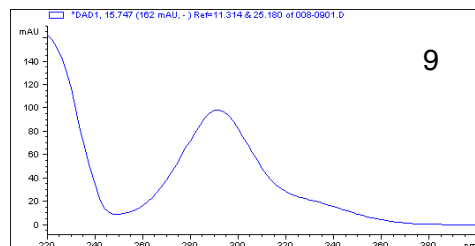
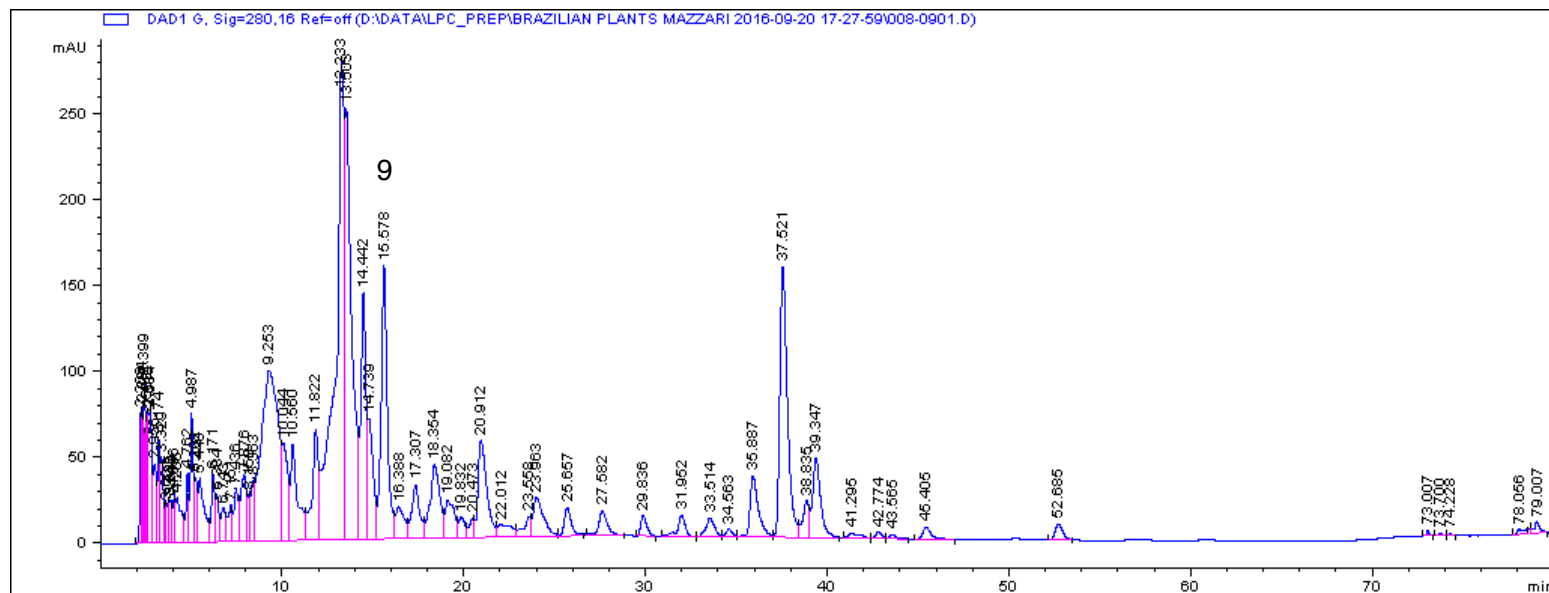


4,5 Flavonoids



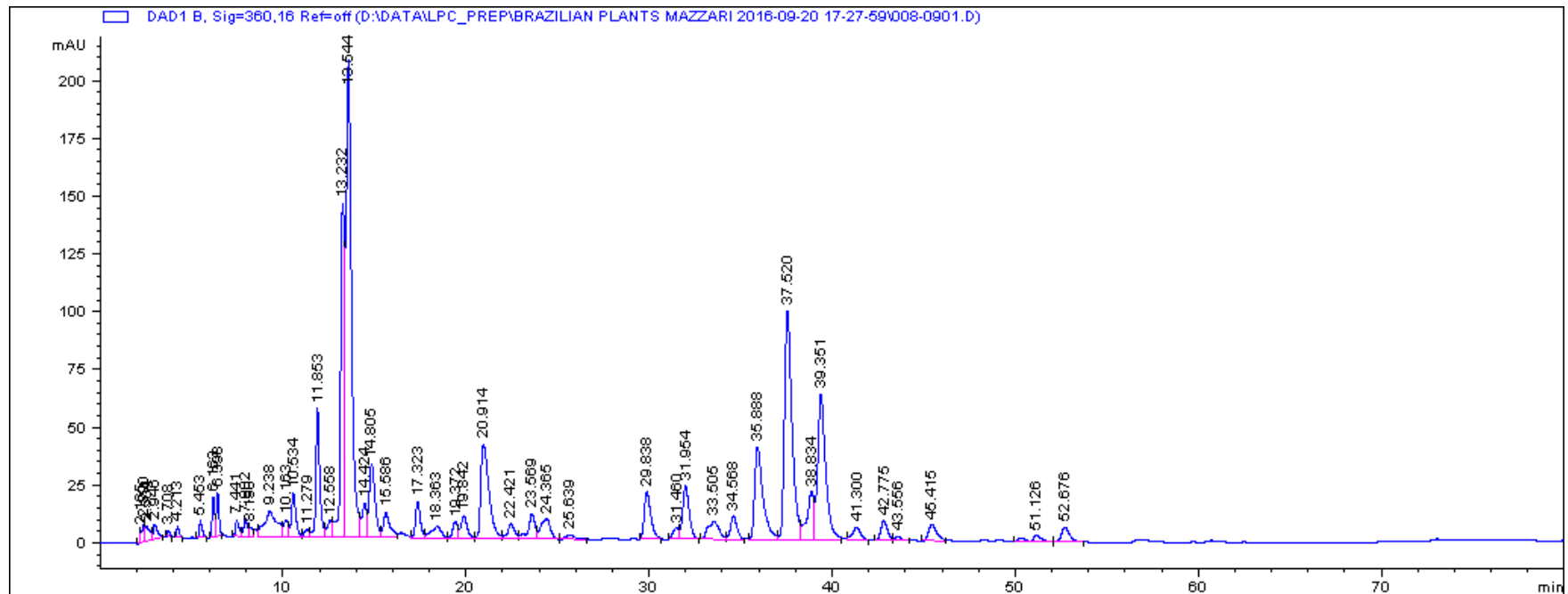
6,7,8 Not identified

Malva sylvestris (flower) 280nm

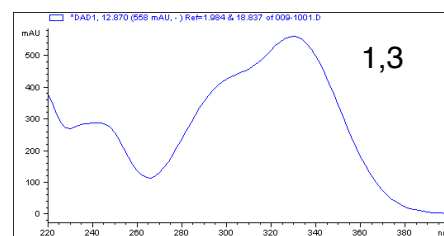
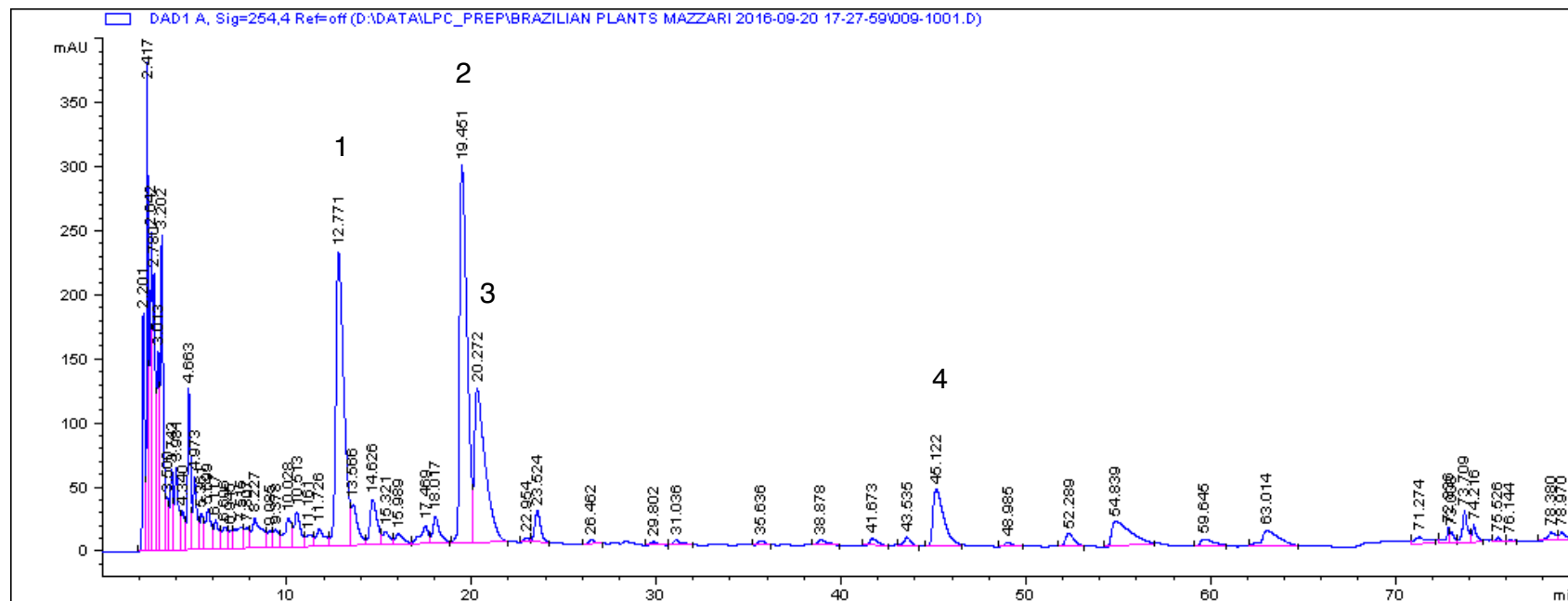


9 Not identified

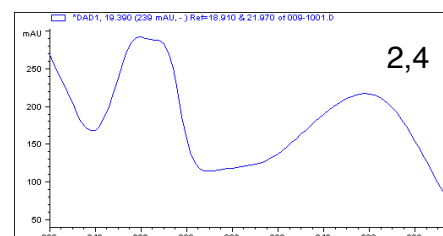
Malva sylvestris (flower) 360nm



Malva sylvestris (herb) 254nm

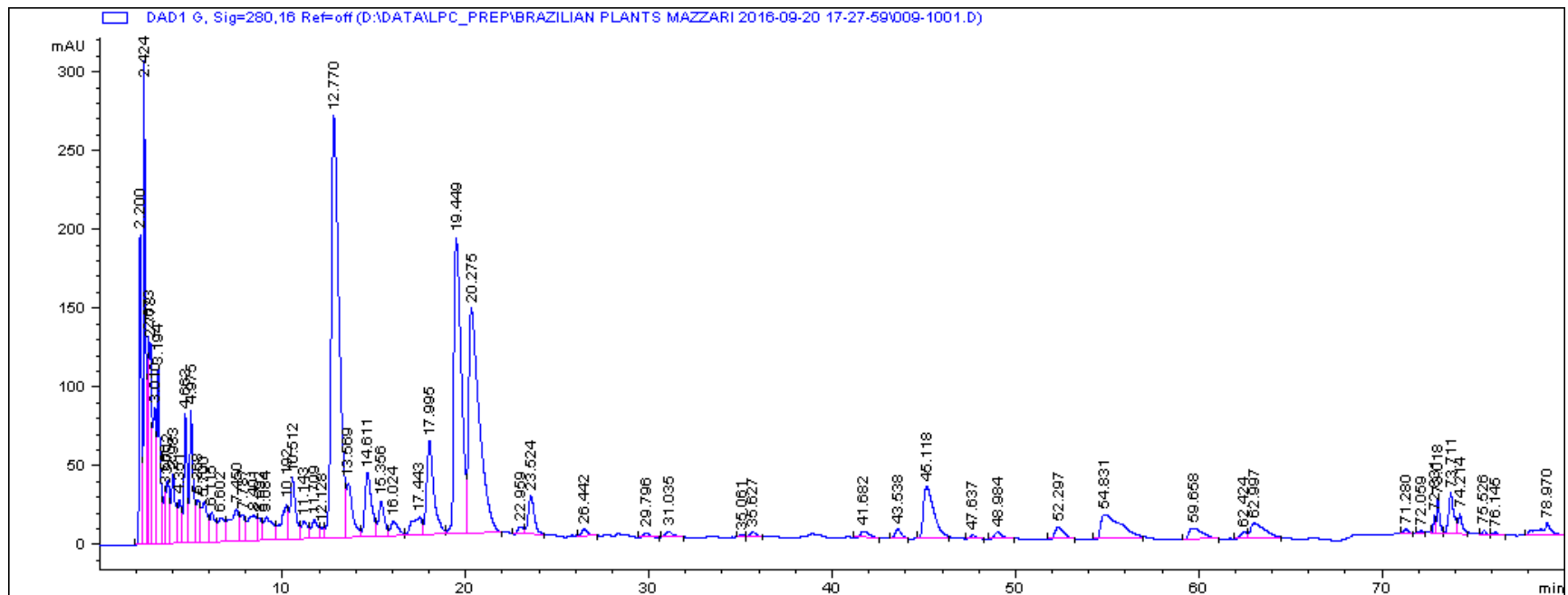


1,3 Phenolic acids

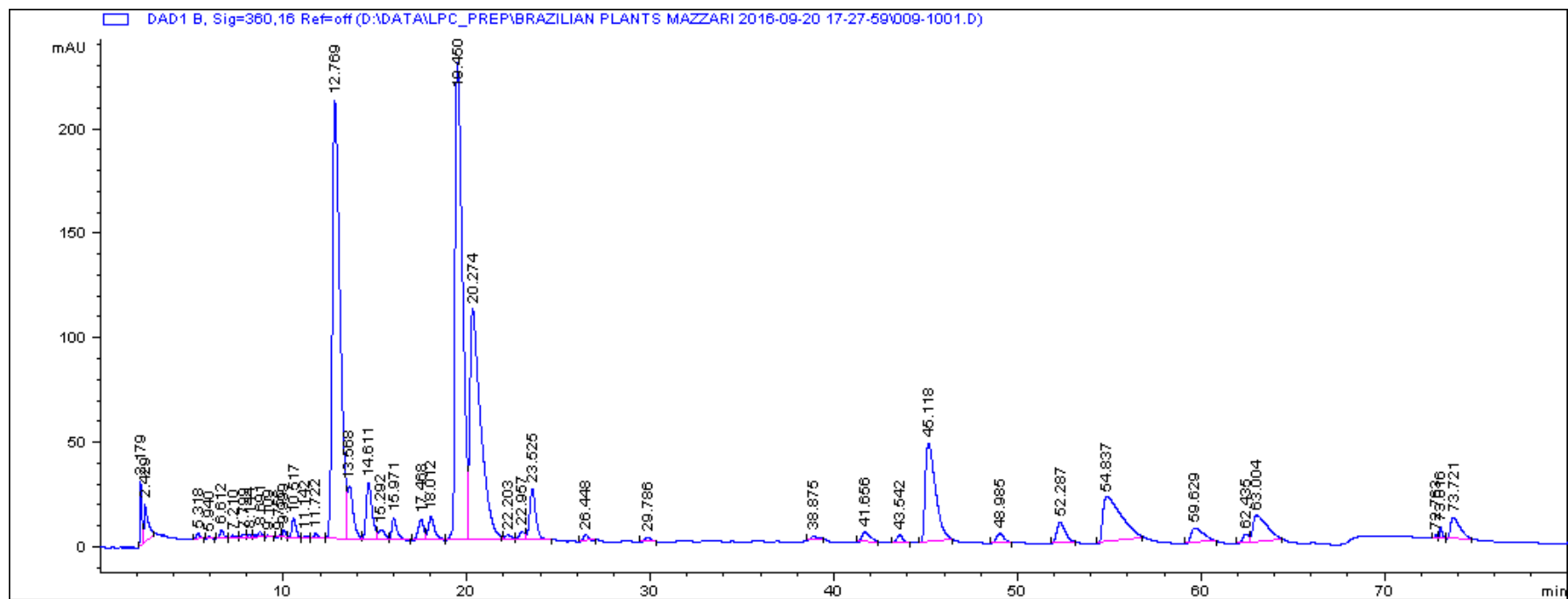


2,4 Flavonoids

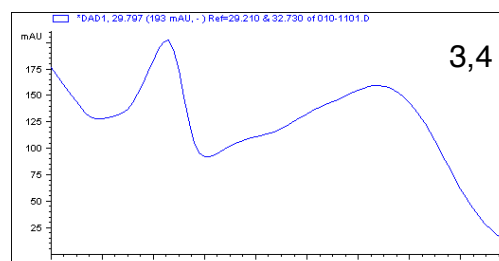
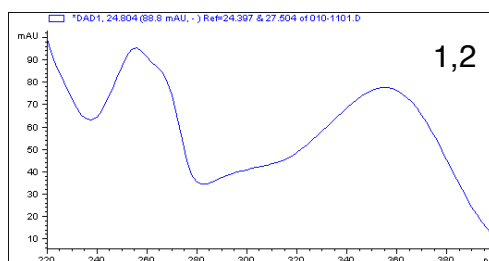
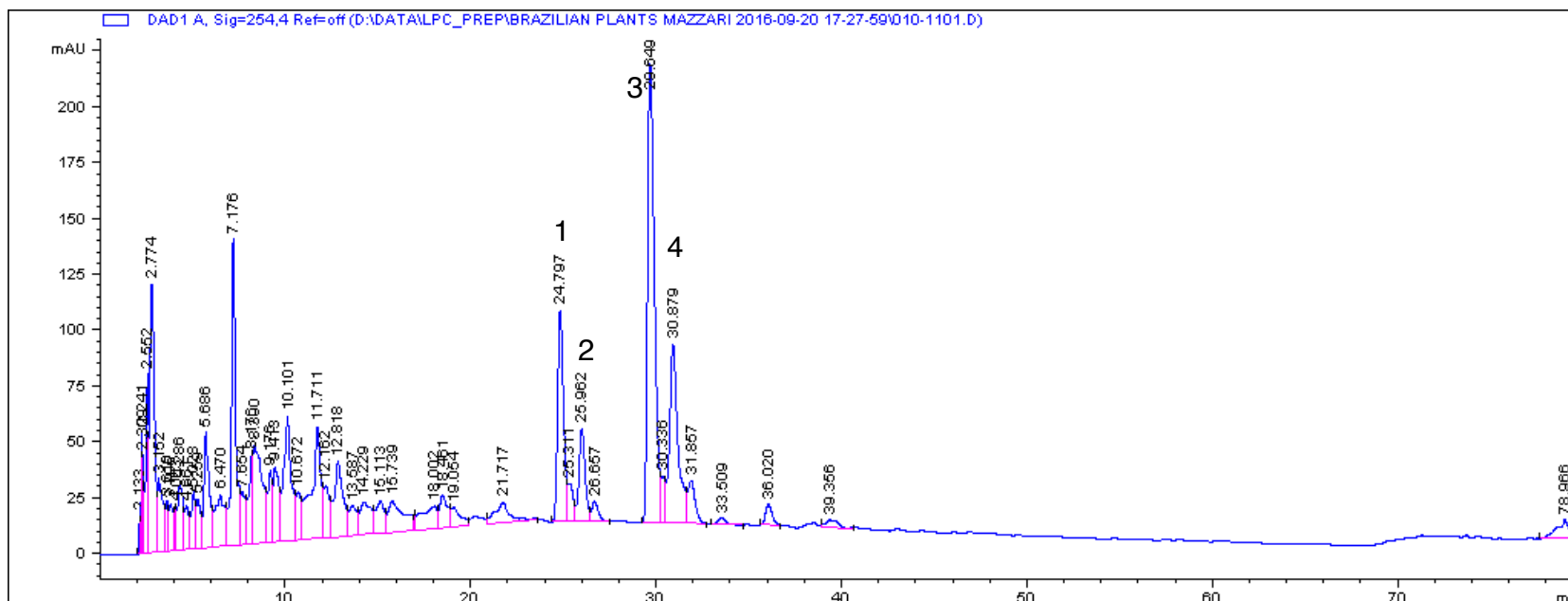
Malva sylvestris (herb) 280nm



Malva sylvestris (herb) 360nm

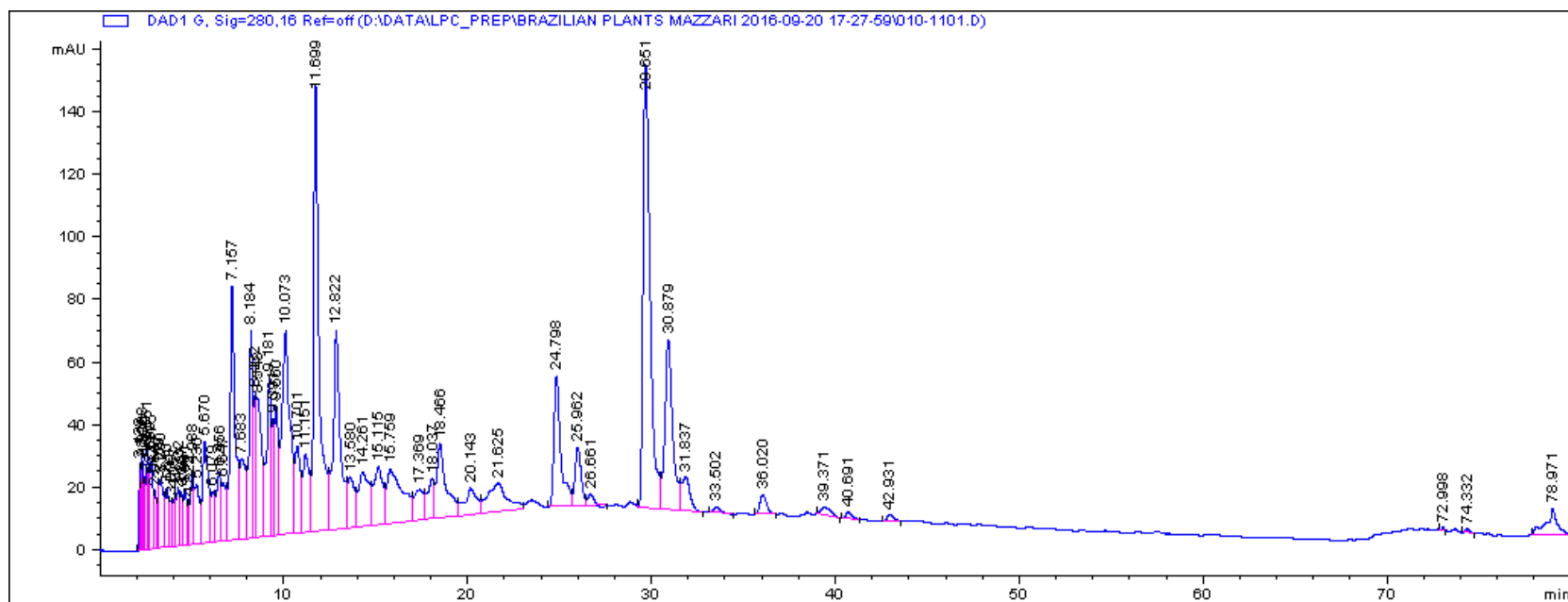


Maytenus ilicifolia 254nm

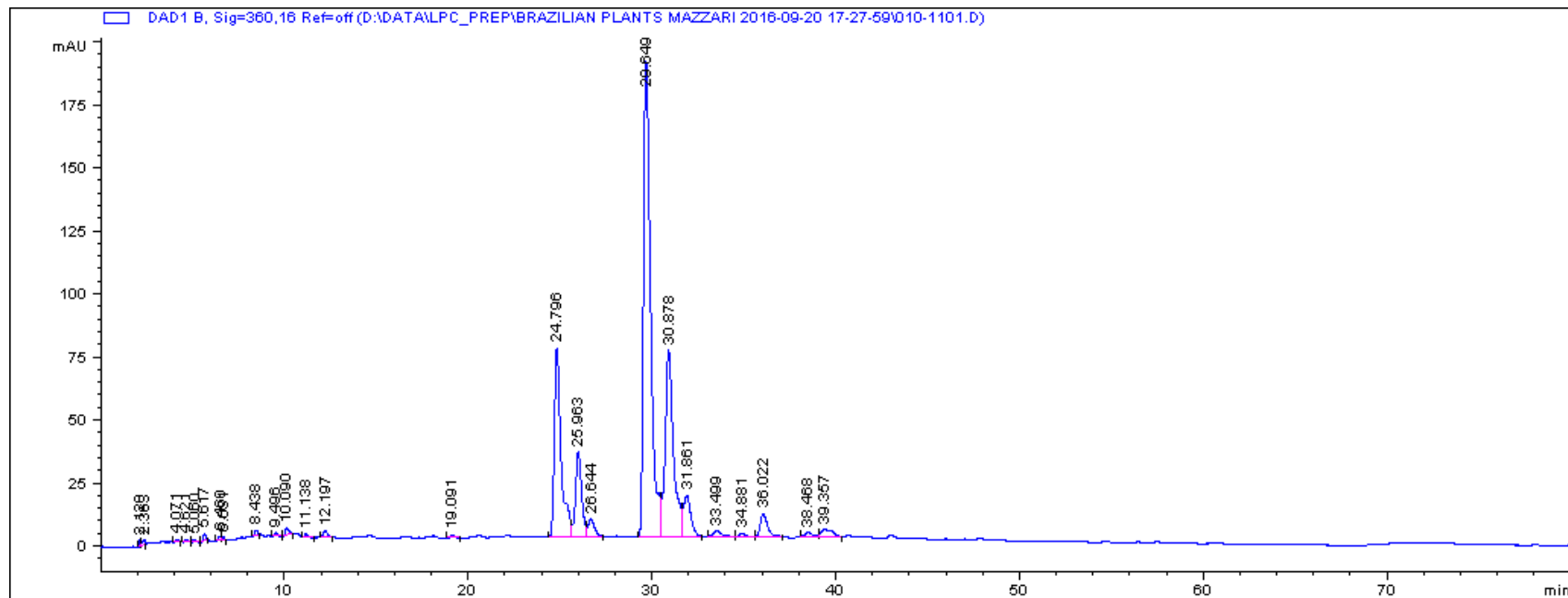


1-4 Glycosilated flavonoids

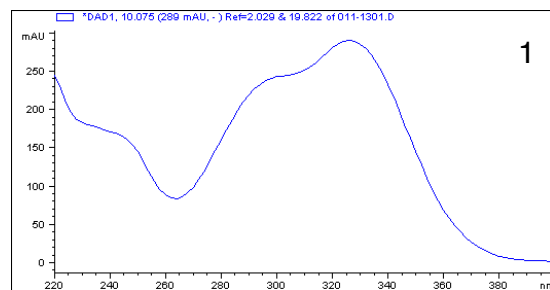
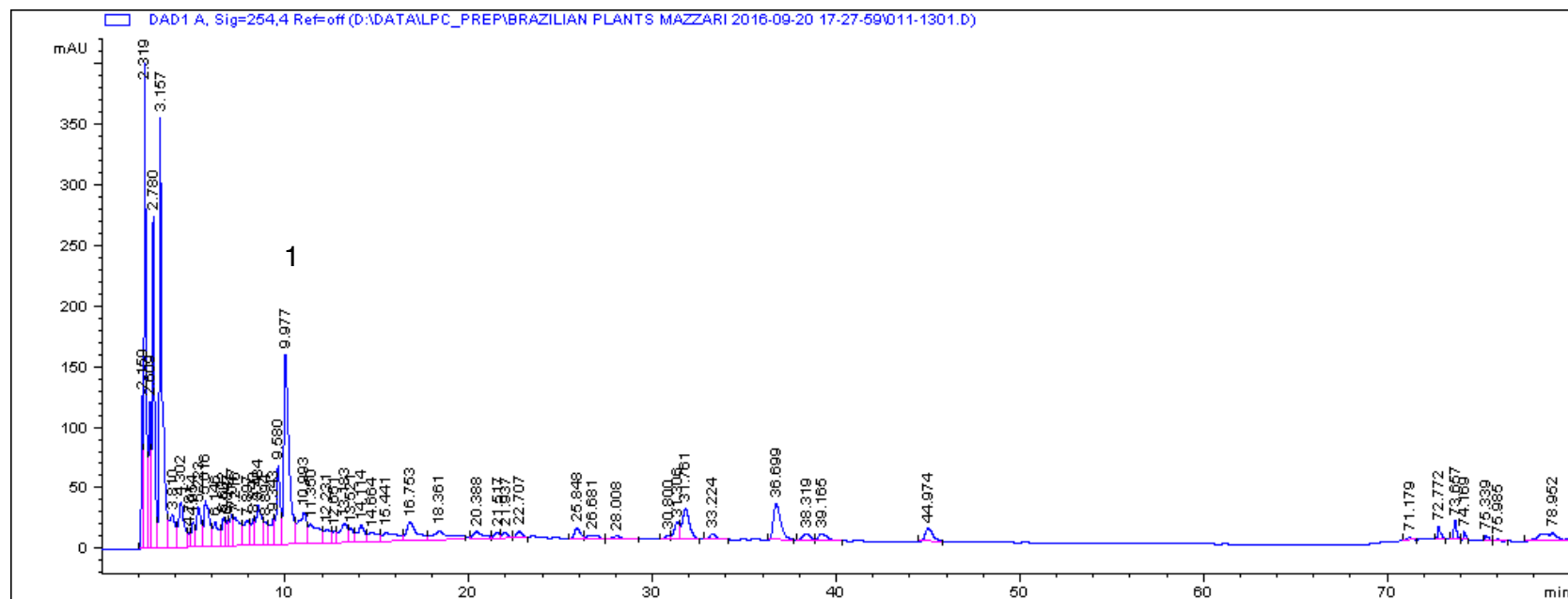
Maytenus ilicifolia 280nm



Maytenus ilicifolia 360nm

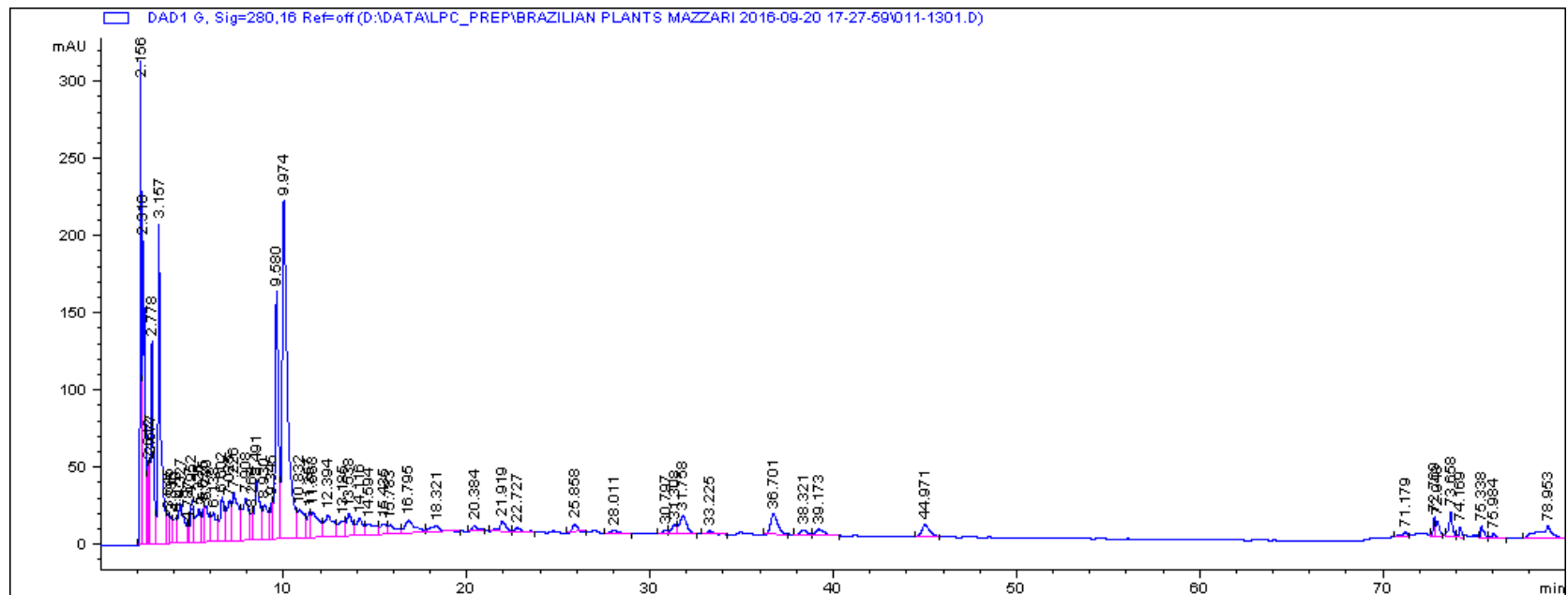


Morus nigra 254nm

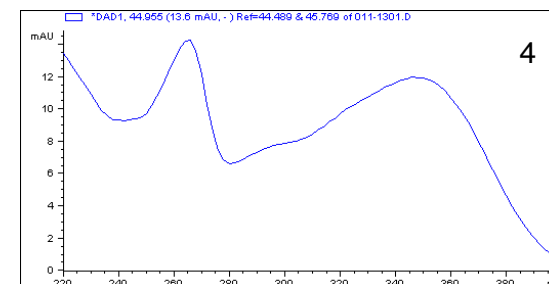
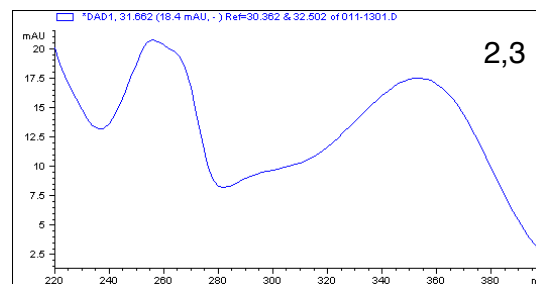
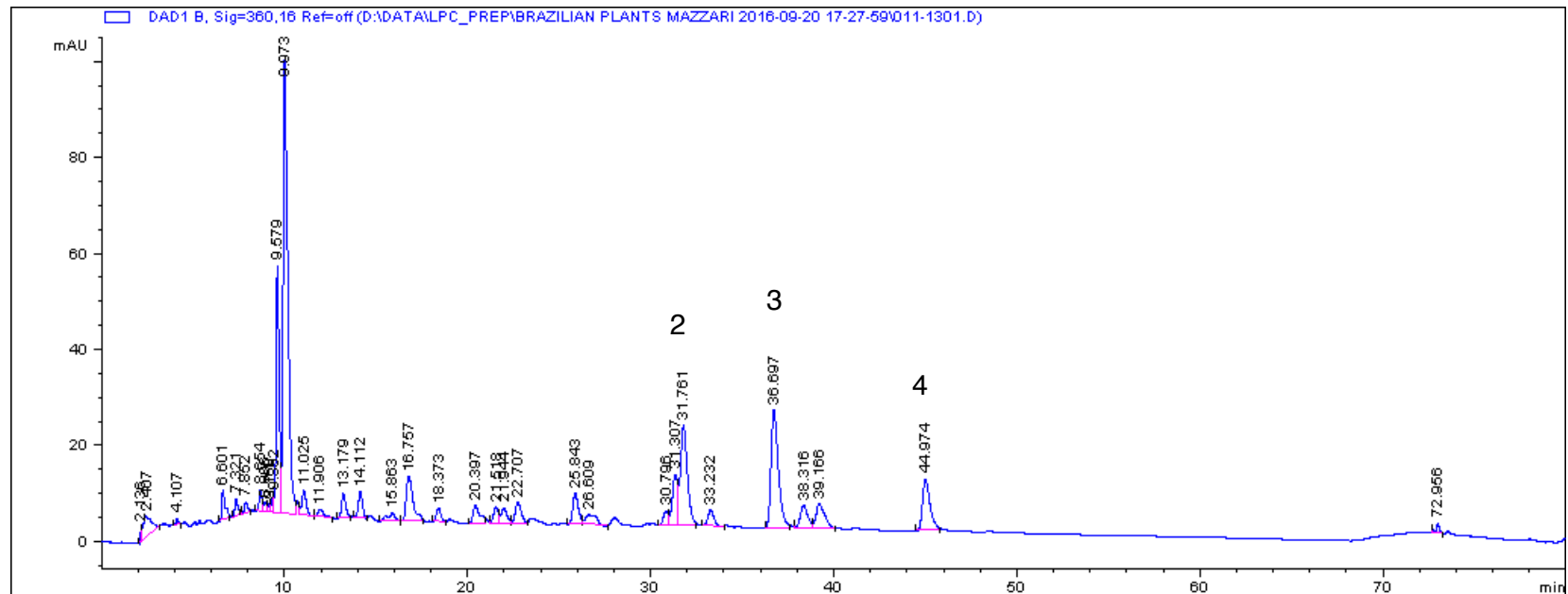


1 Chlorogenic acid

Morus nigra 280nm

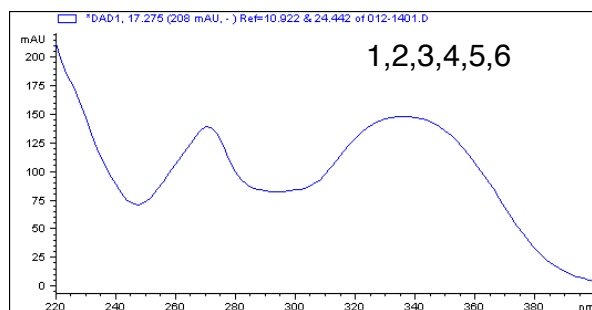
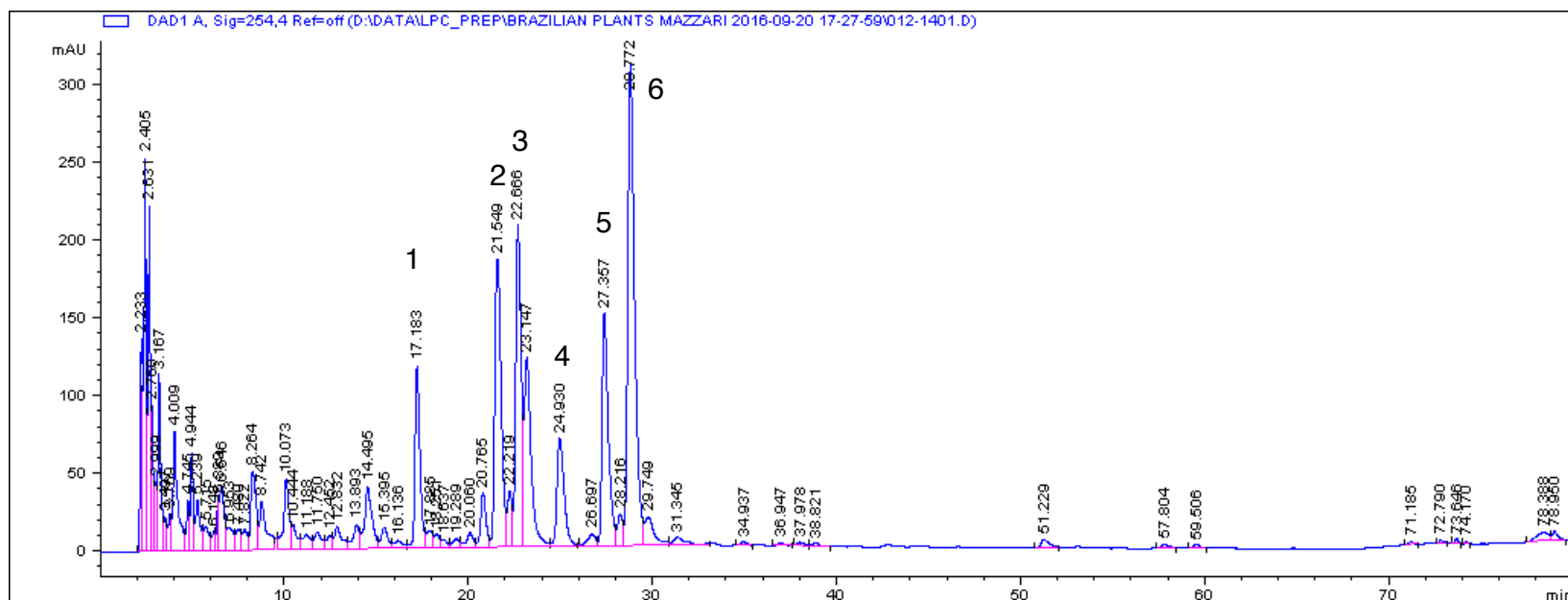


Morus nigra 360nm



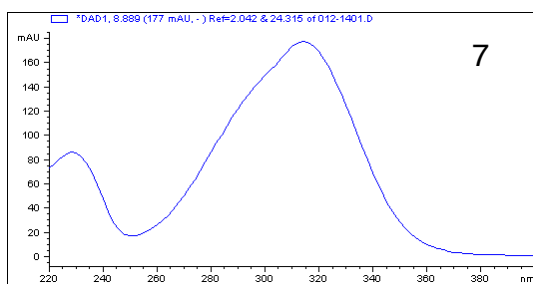
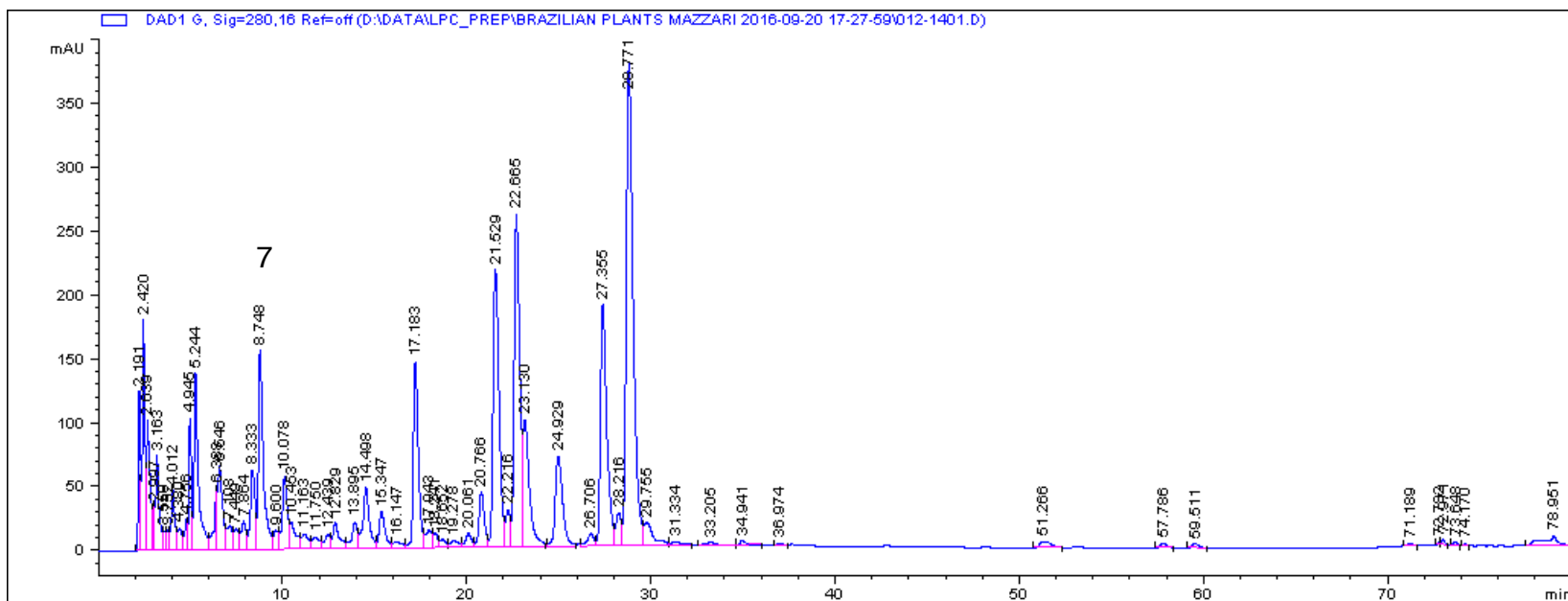
2 - 4 Not identified

Passiflora incarnata 254nm



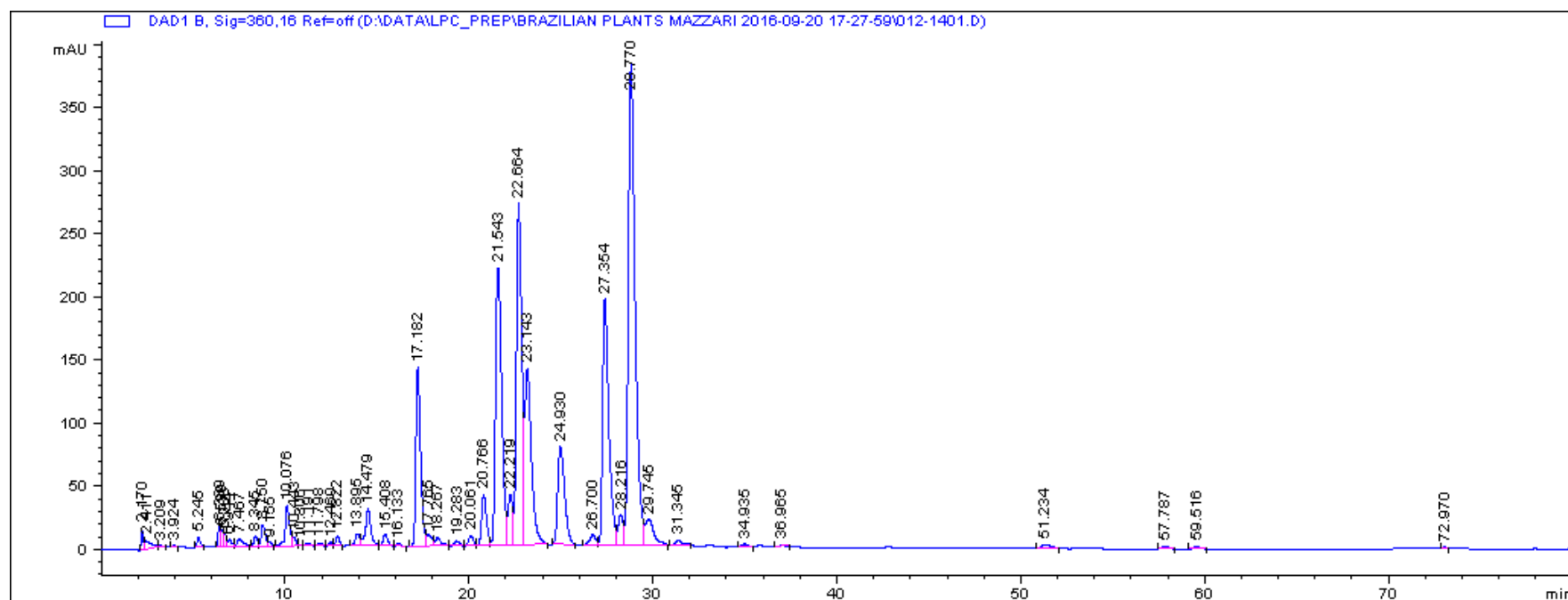
1-6 Glycosilated flavonoids

Passiflora incarnata 280nm

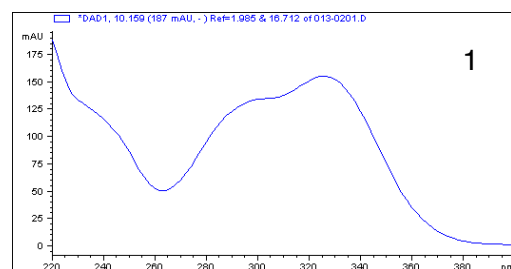
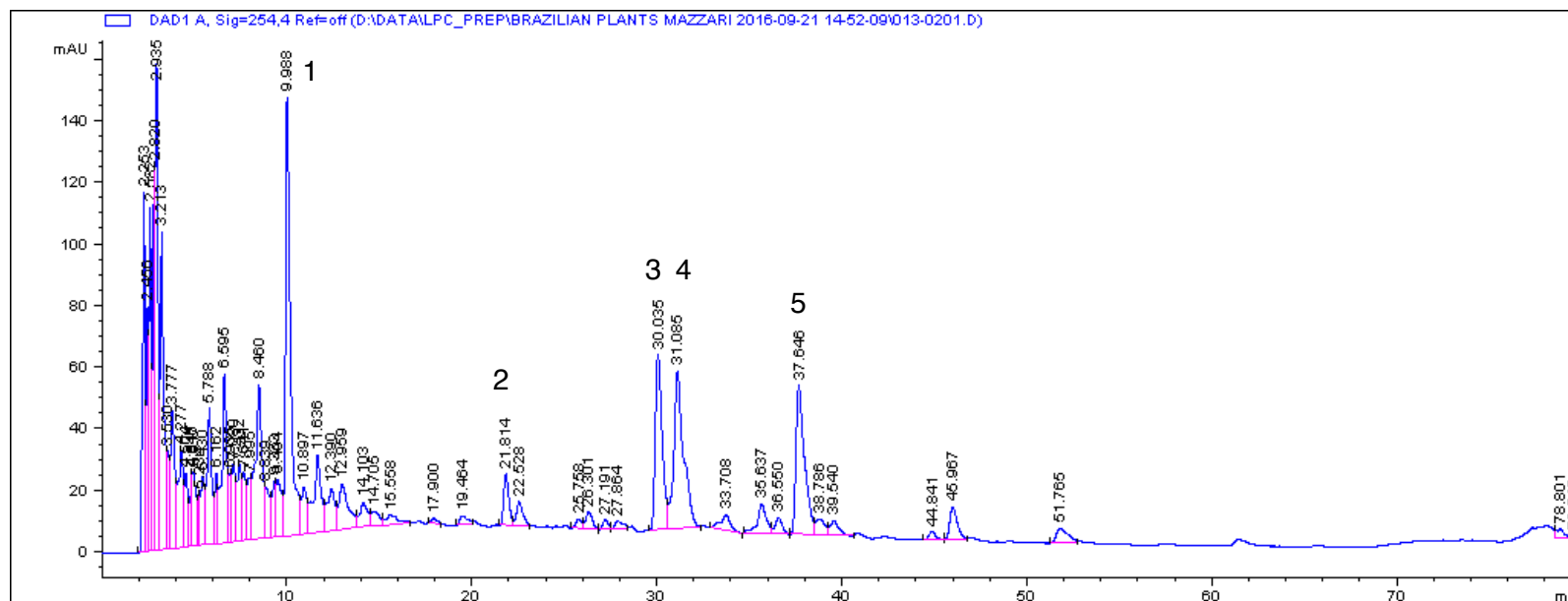


7 Not identified

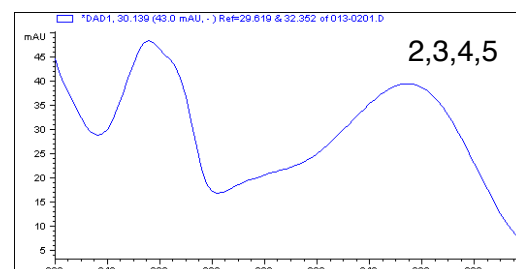
Passiflora incarnata 360nm



Persea americana 254nm

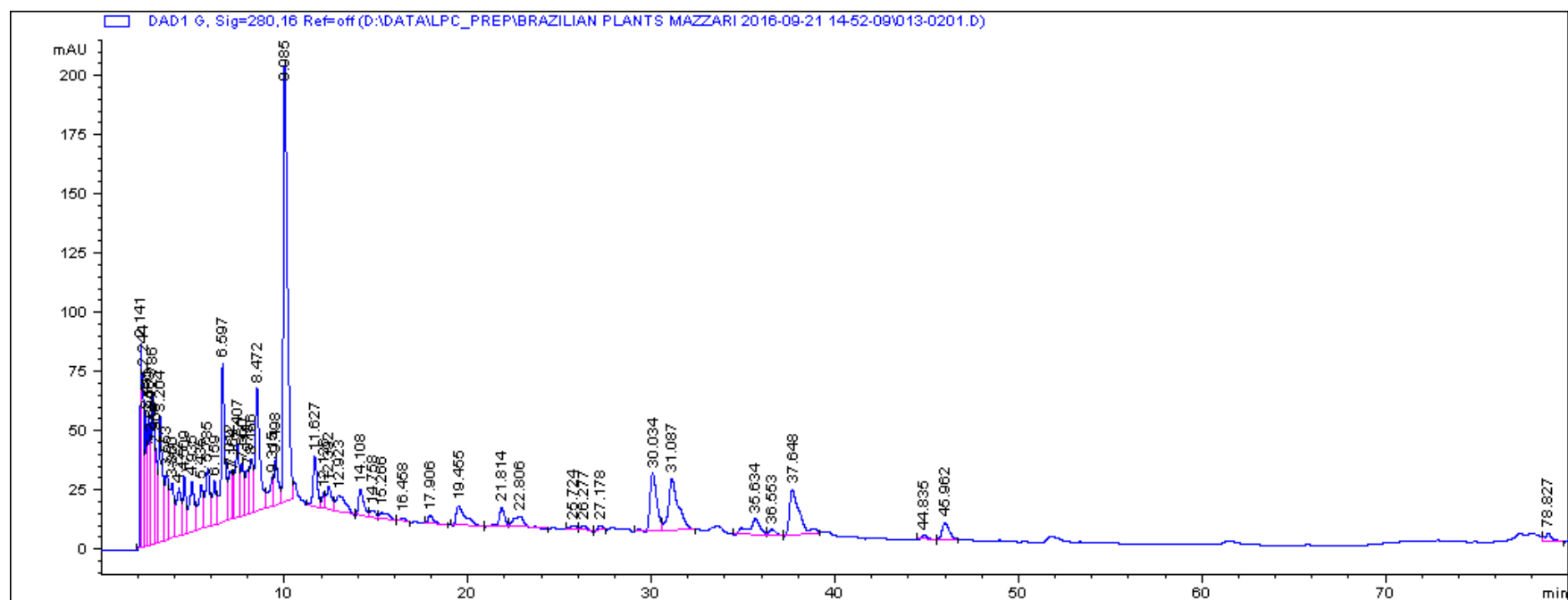


1 Chlorogenic acid

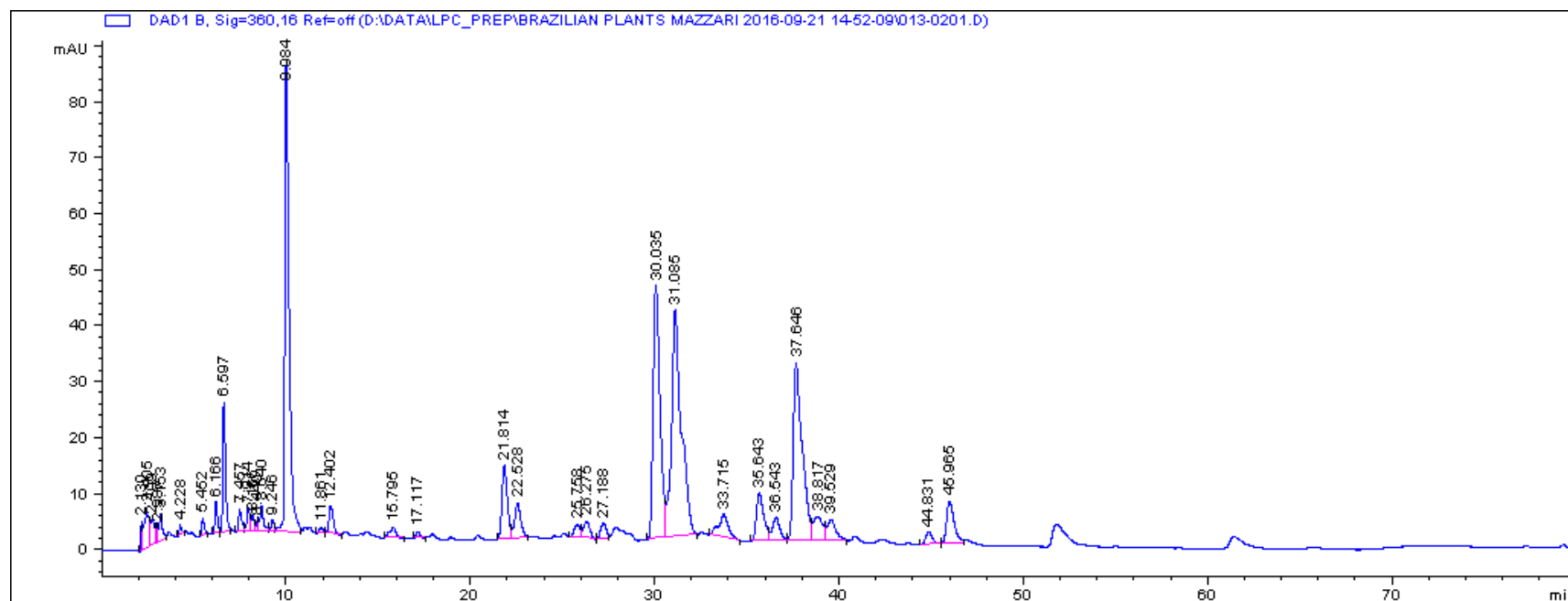


2,3,4,5 Glycosilated flavonoids

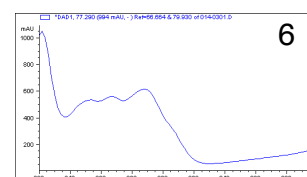
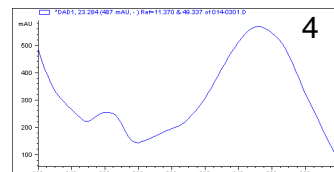
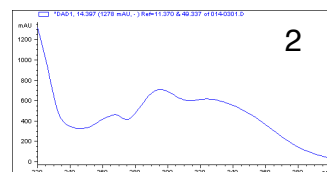
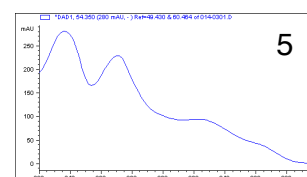
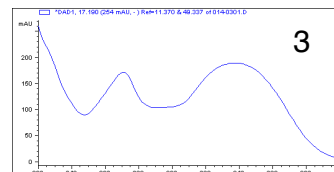
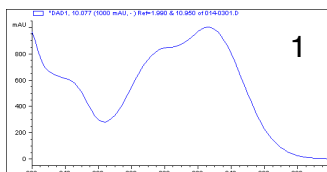
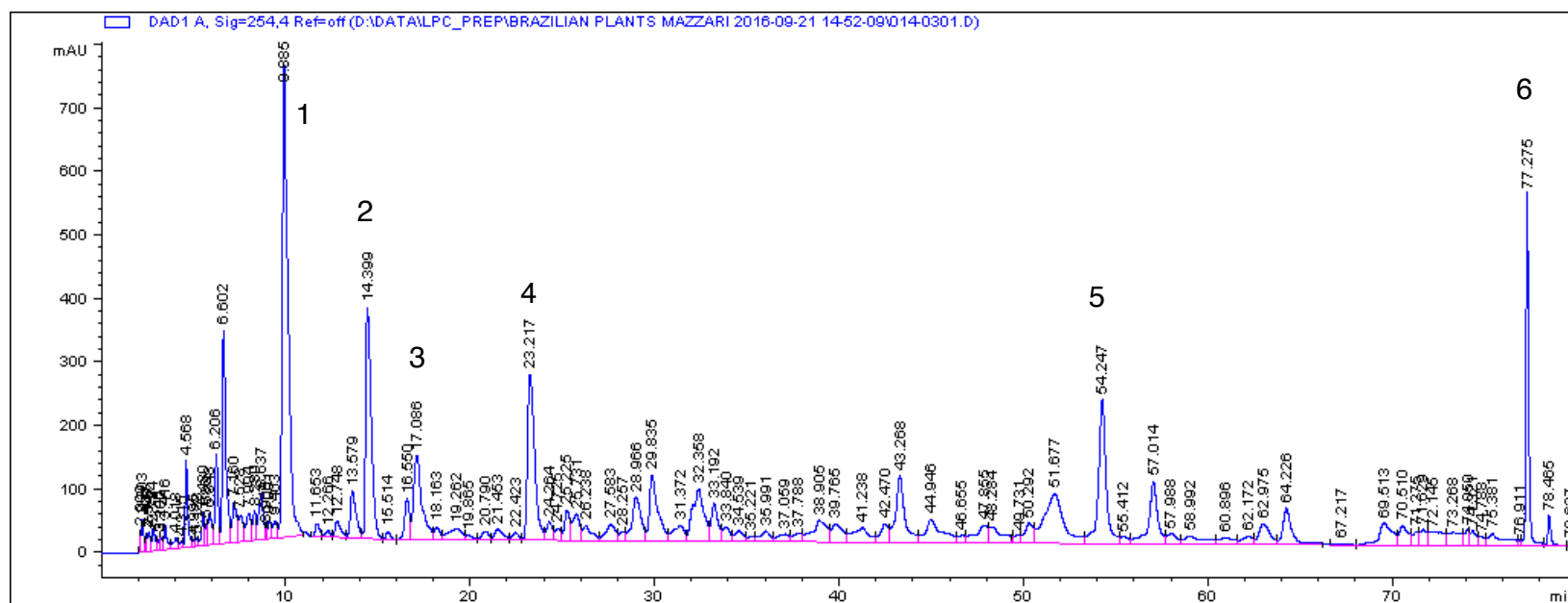
Persea americana 280nm



Persea americana 360nm



Rhamnus purshiana 254nm

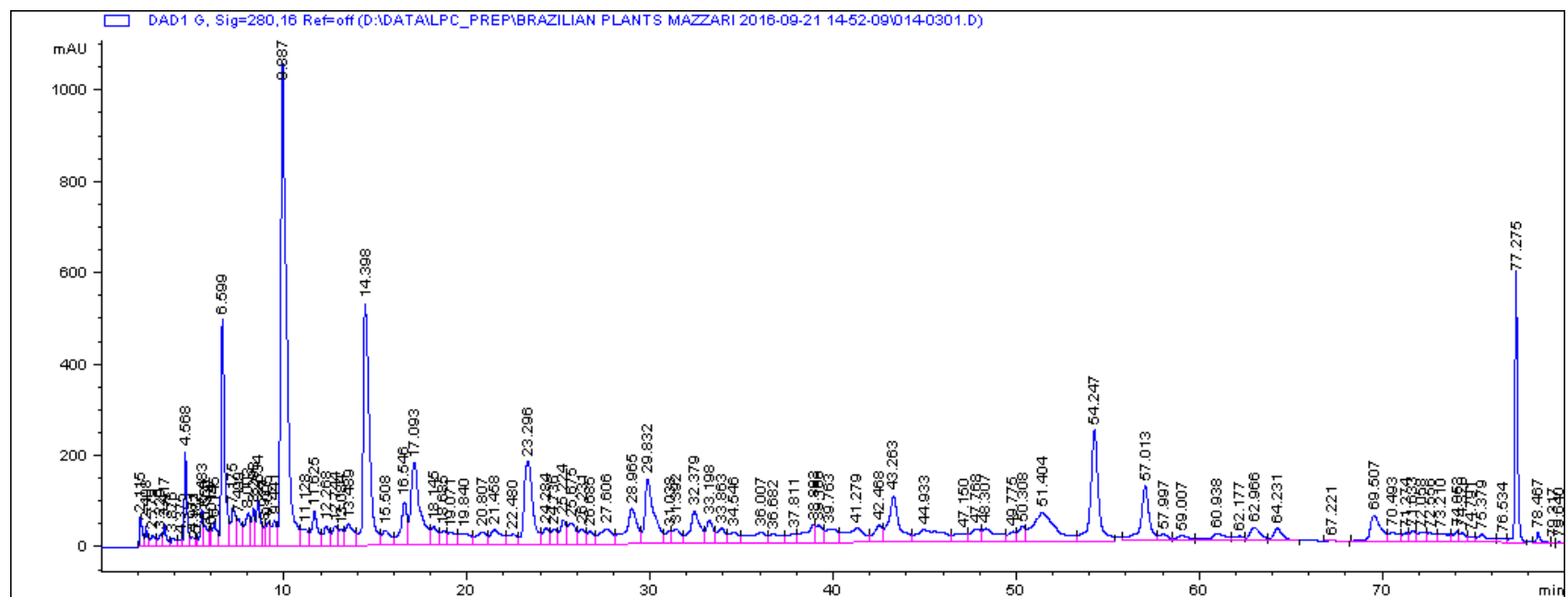


1 Chlorogenic acid

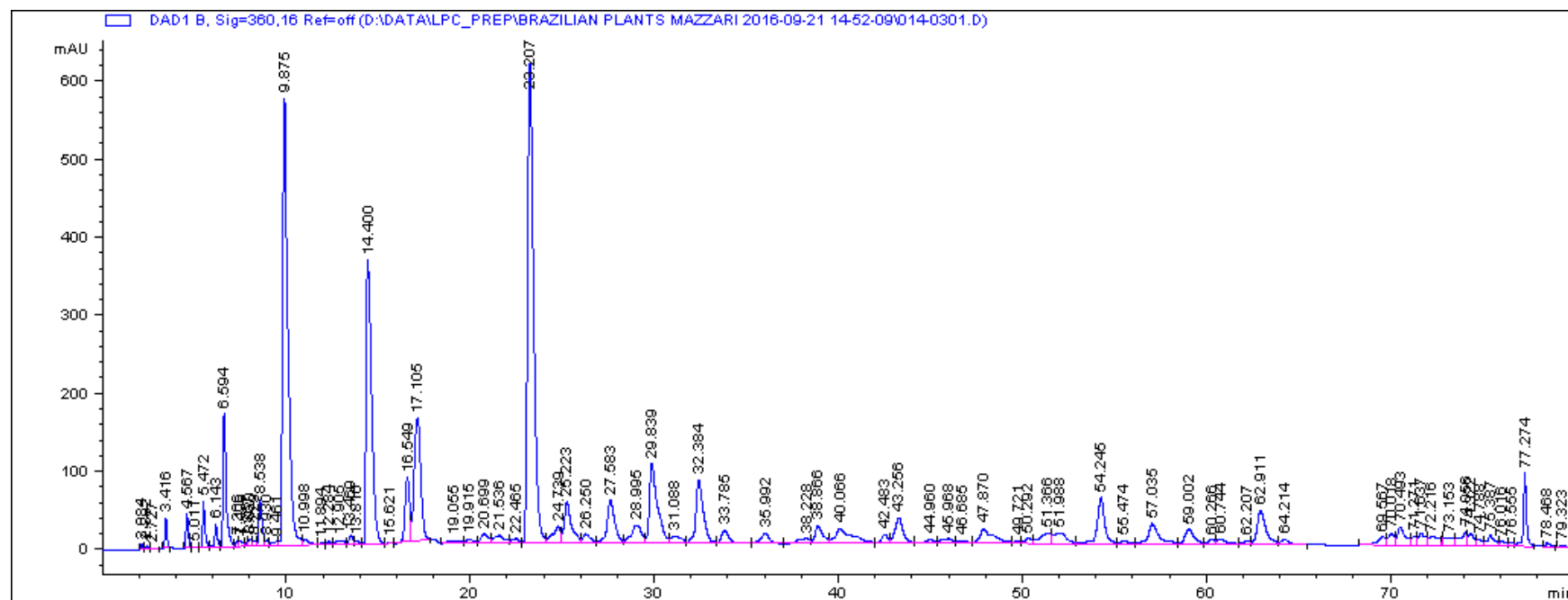
3 Glycosilated flavonoid

2,4,5,6 Not identified

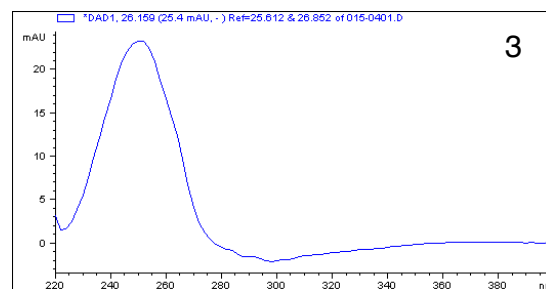
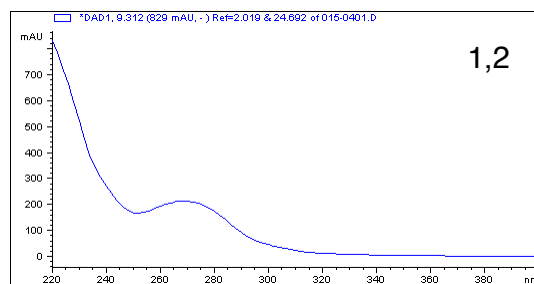
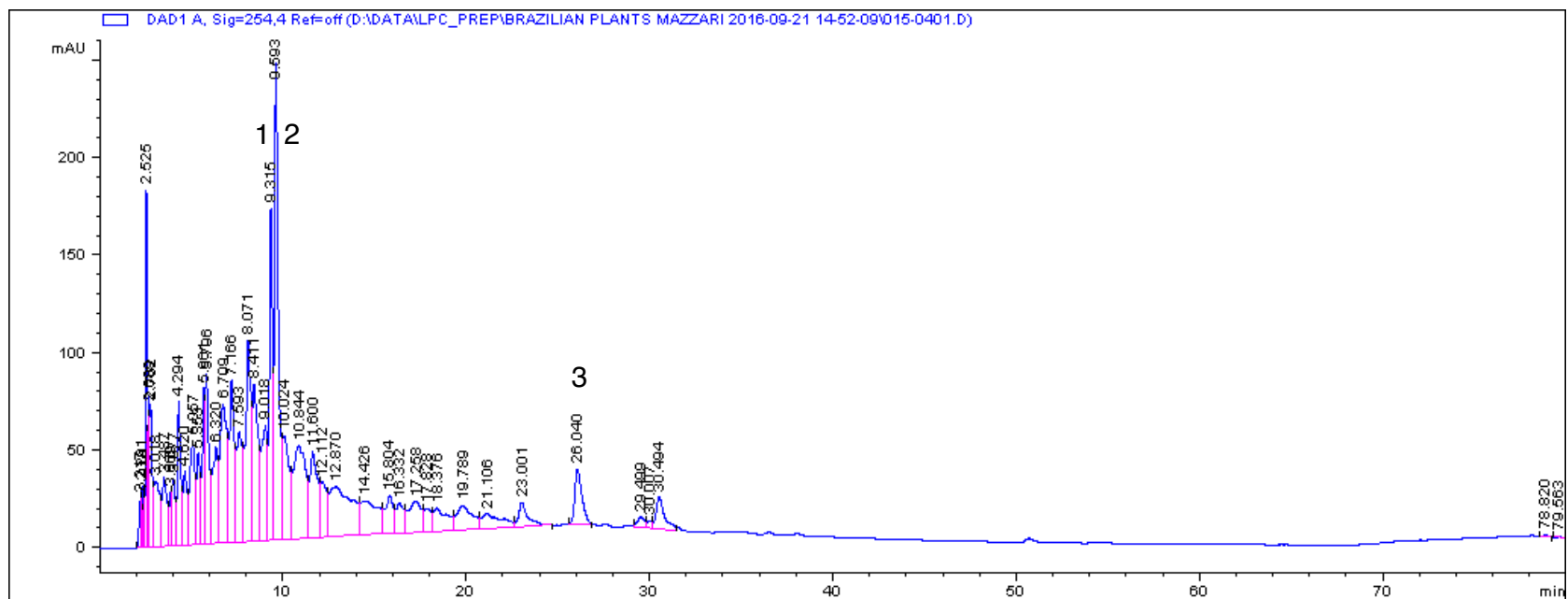
Rhamnus purshiana 280nm



Rhamnus purshiana 360nm

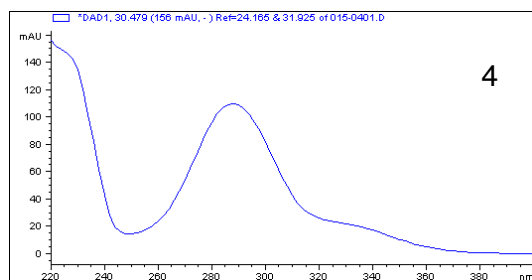
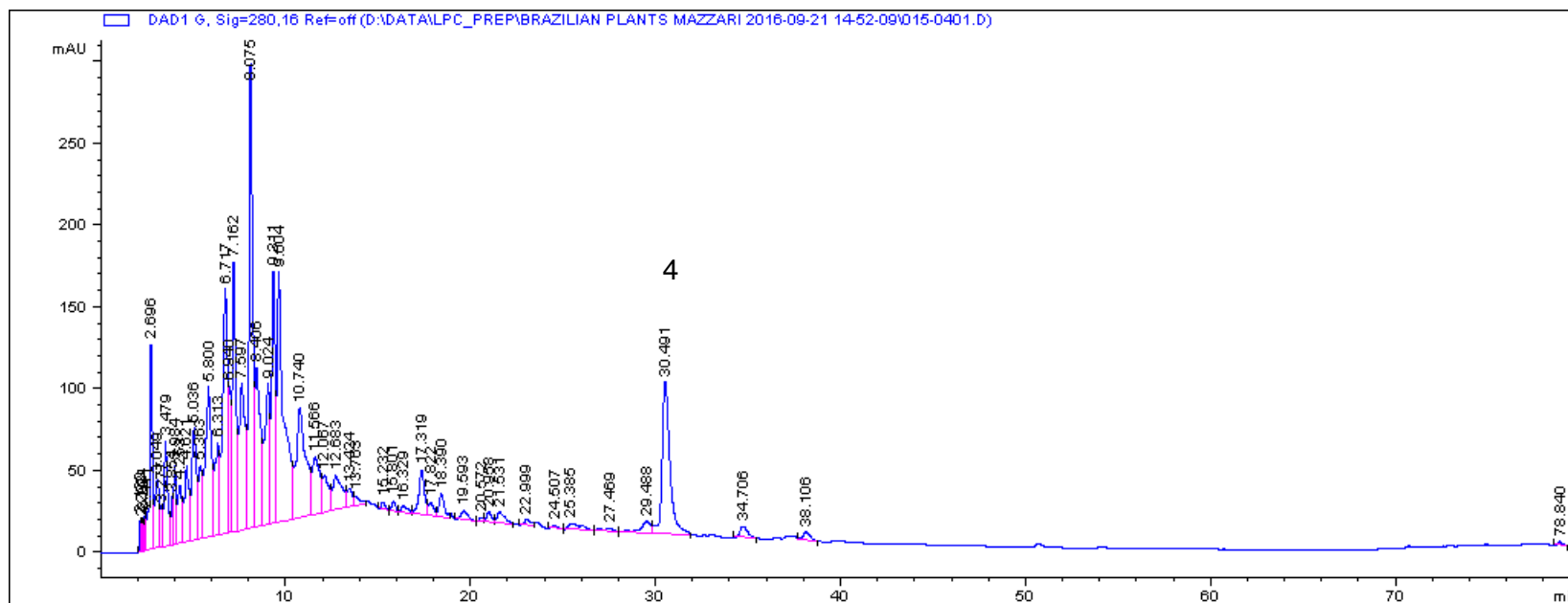


Salix alba 254nm



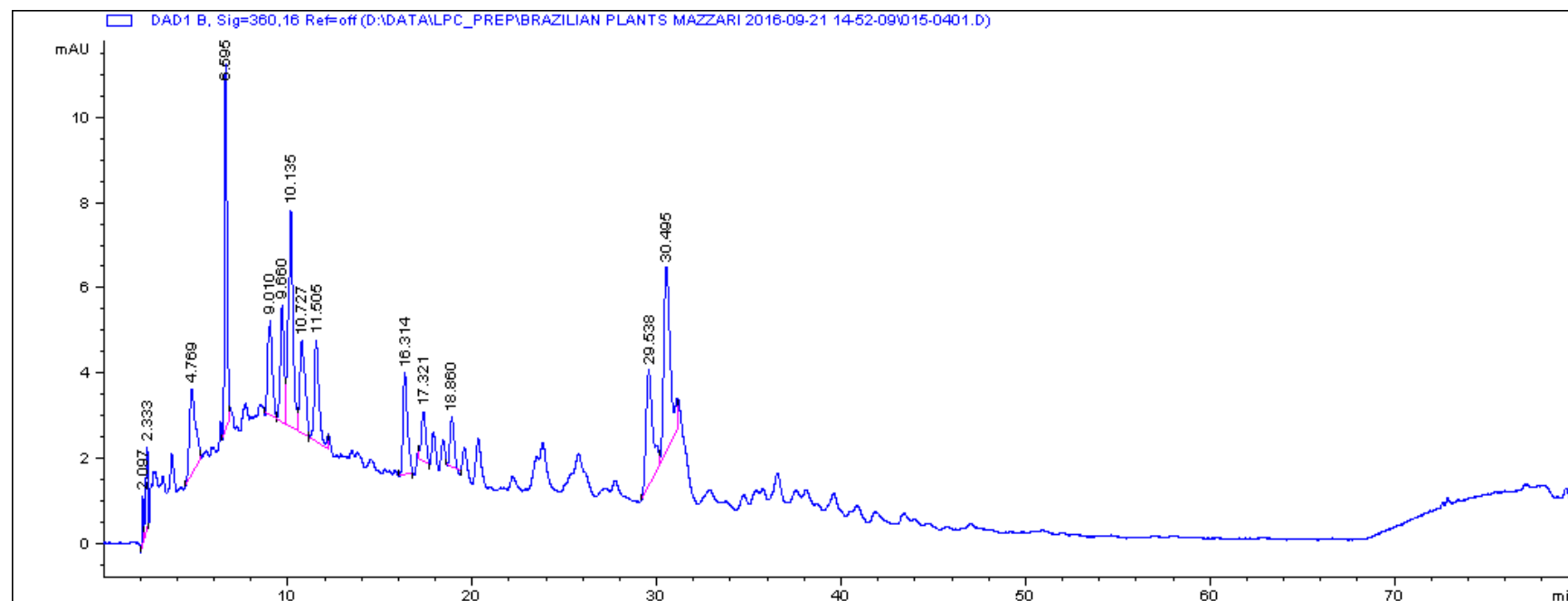
1-3 Not identified

Salix alba 280nm

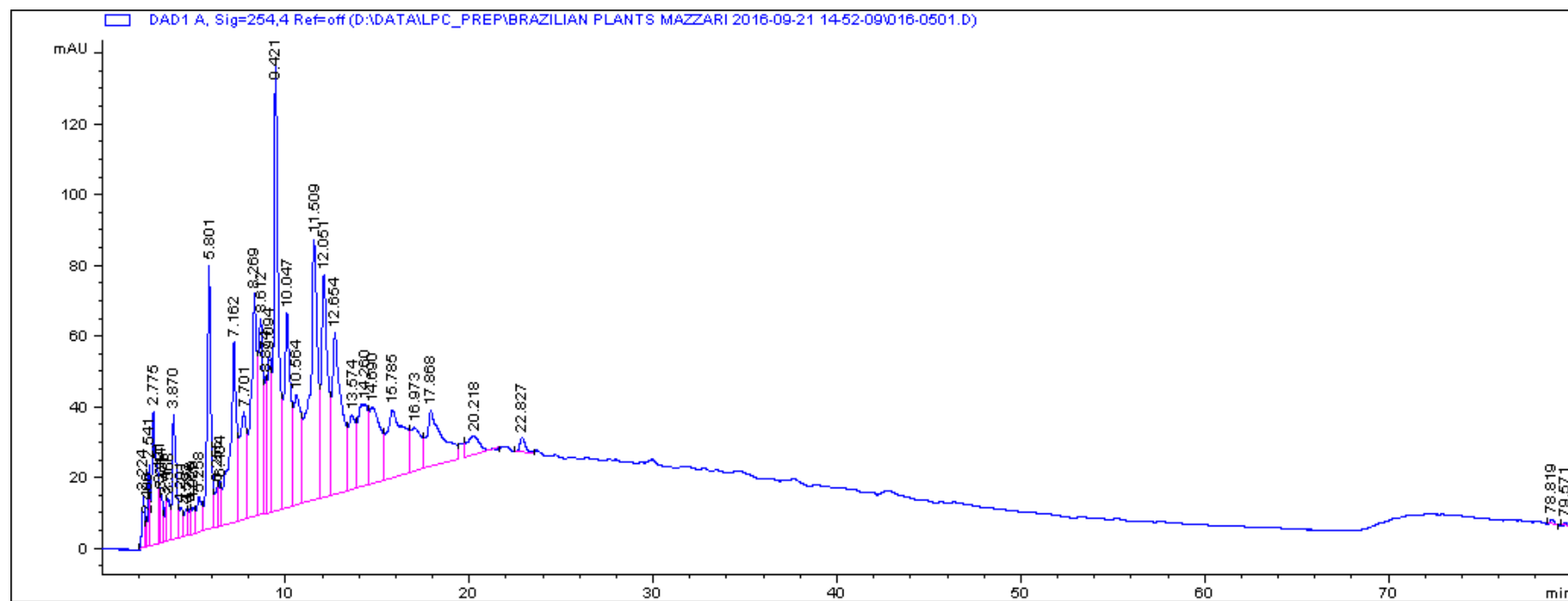


4 Not identified

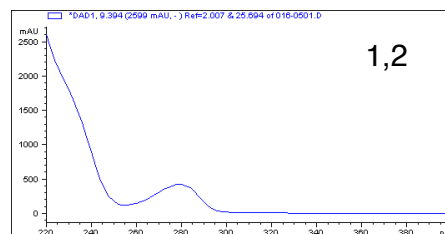
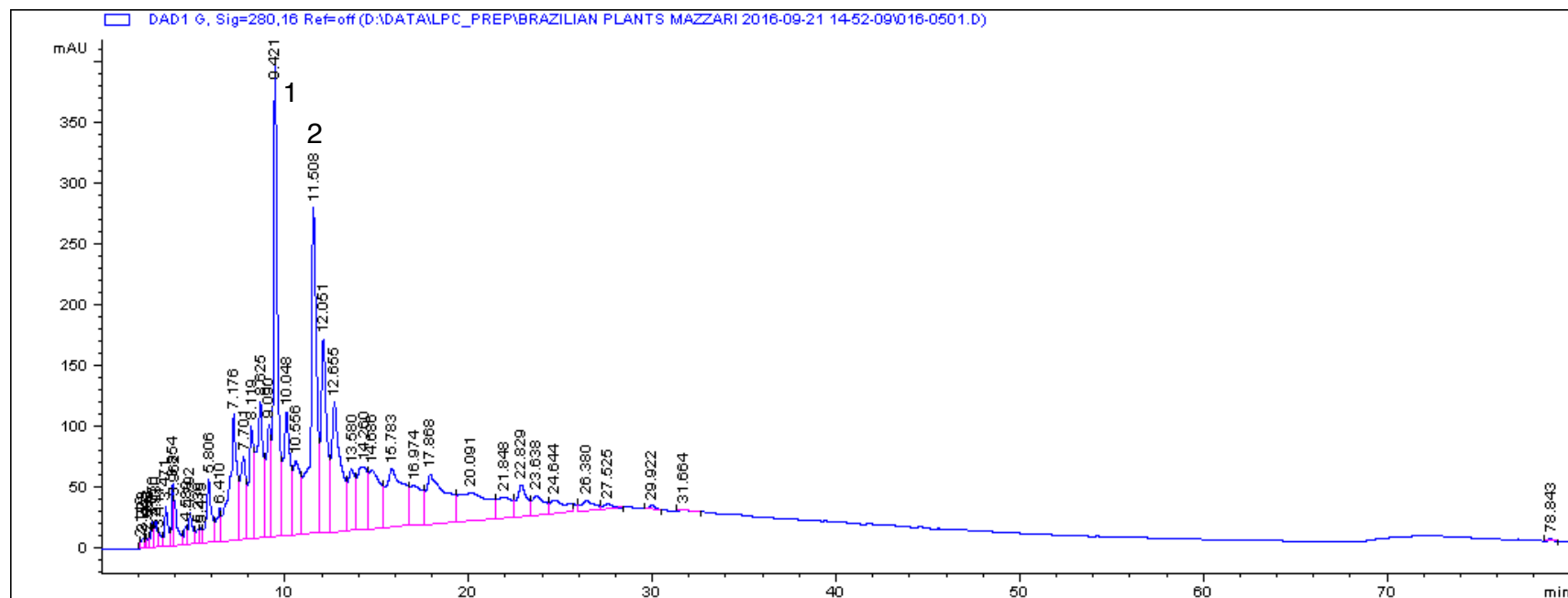
Salix alba 360nm



Schinus terebinthifolia 254nm

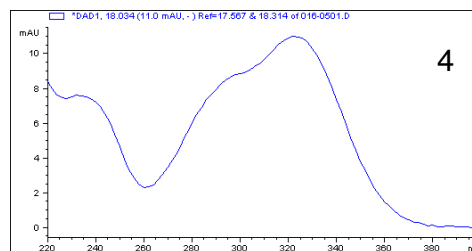
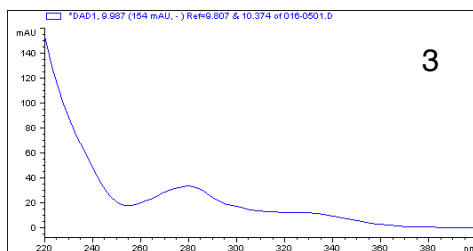
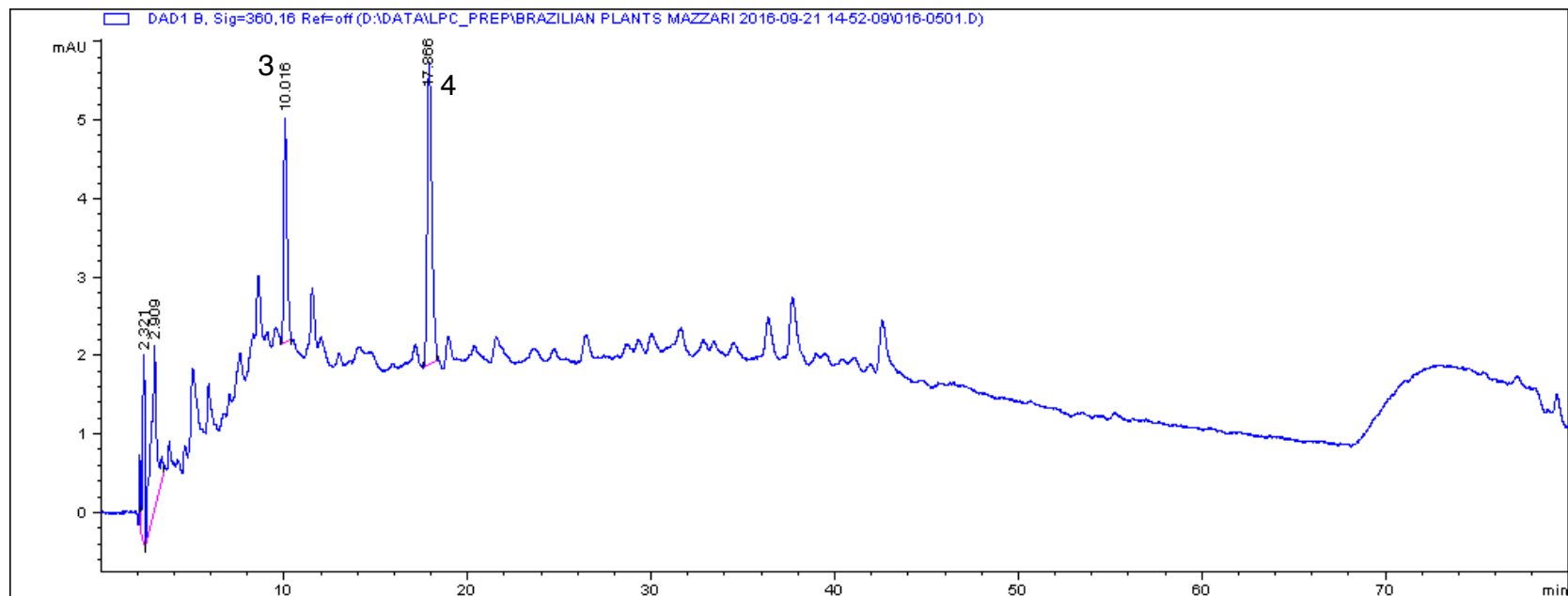


Schinus terebinthifolia 280nm



1,2 Not identified

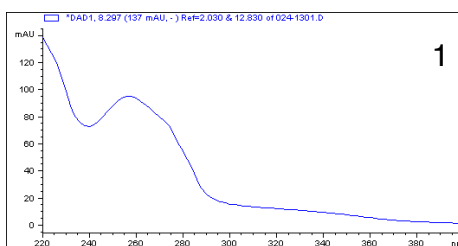
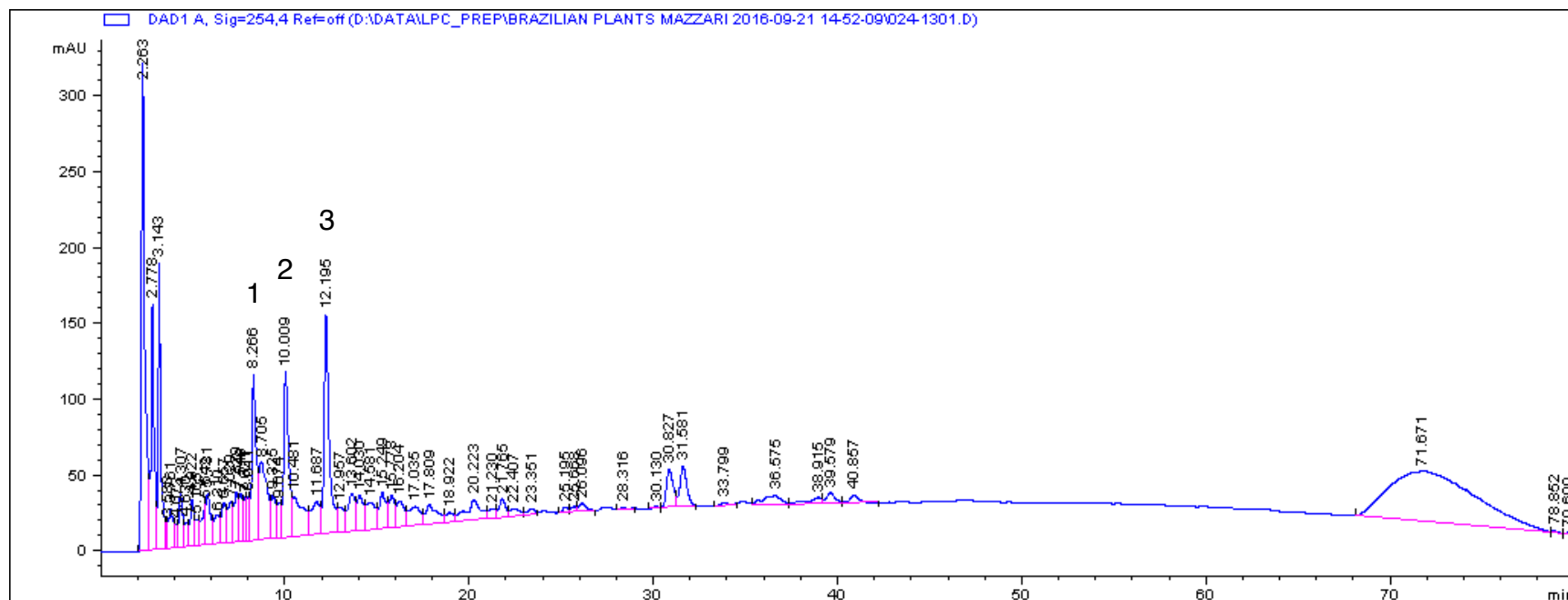
Schinus terebinthifolia 360nm



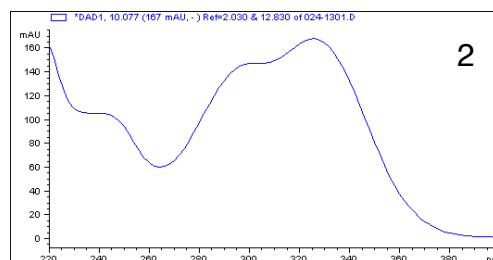
3 Not identified

4 Phenolic acid

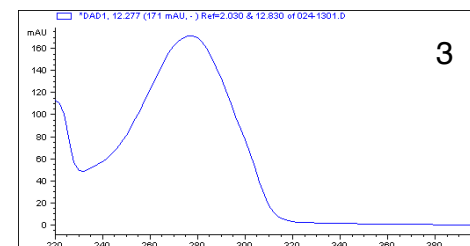
Solanum paniculatum 254nm



1 Not identified

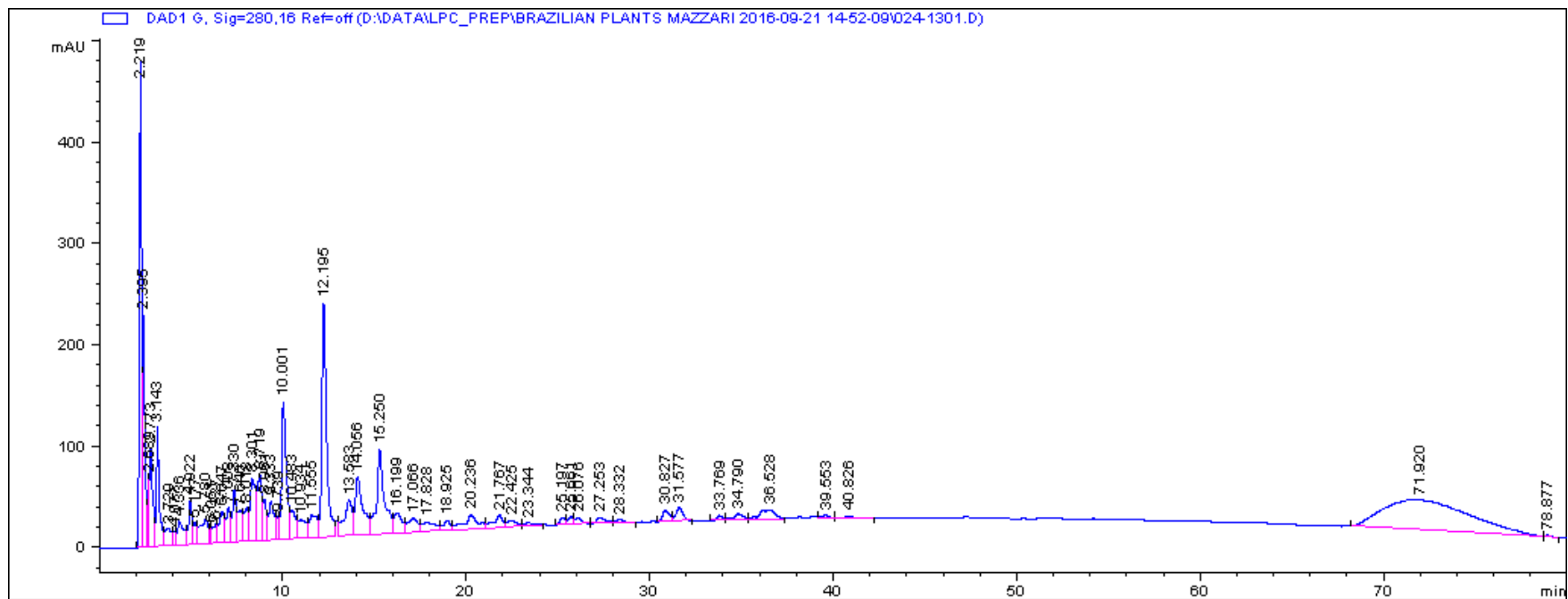


2 Chlorogenic acid

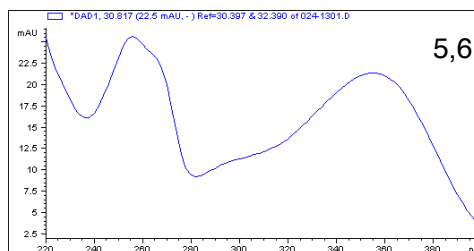
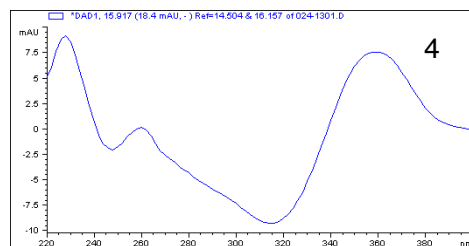
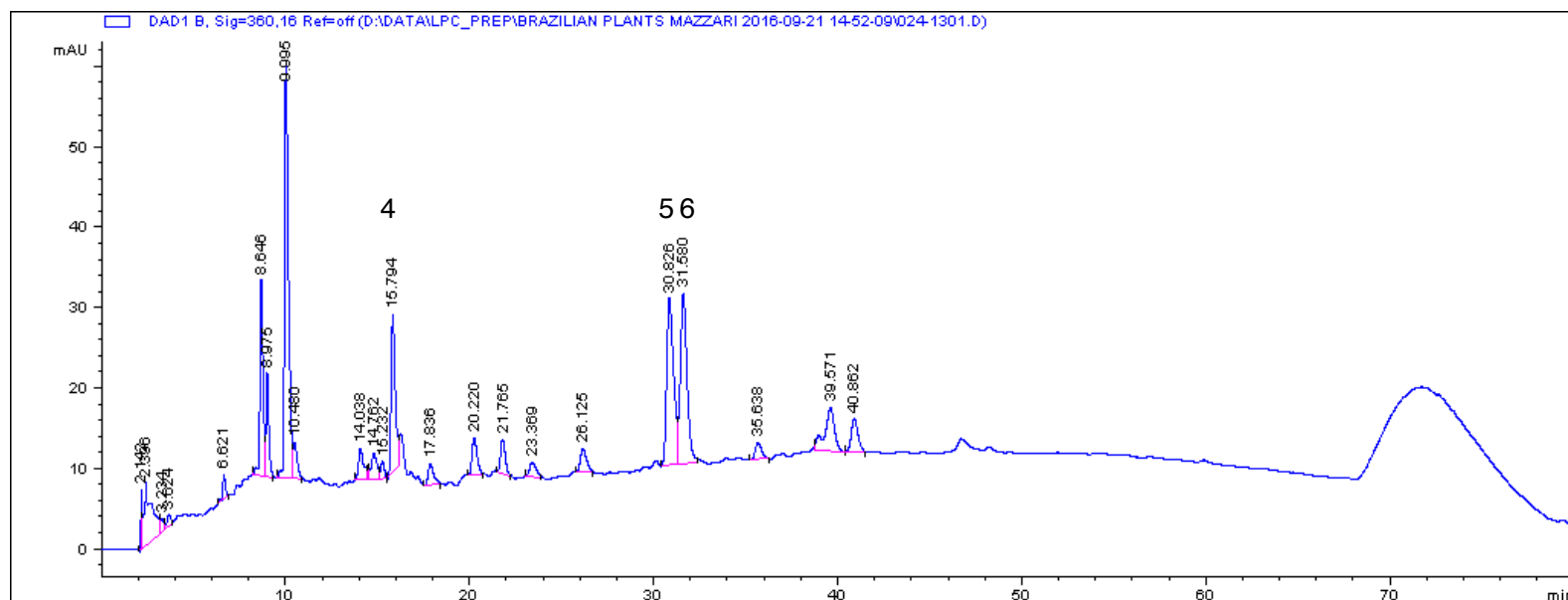


3 Not identified

Solanum paniculatum 280nm



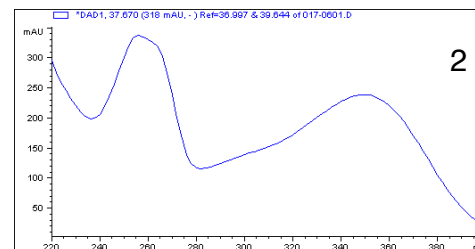
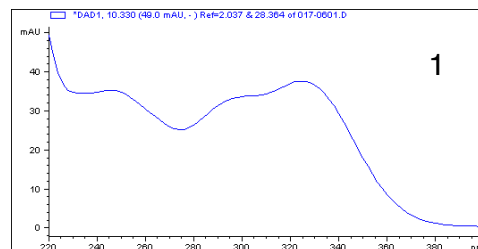
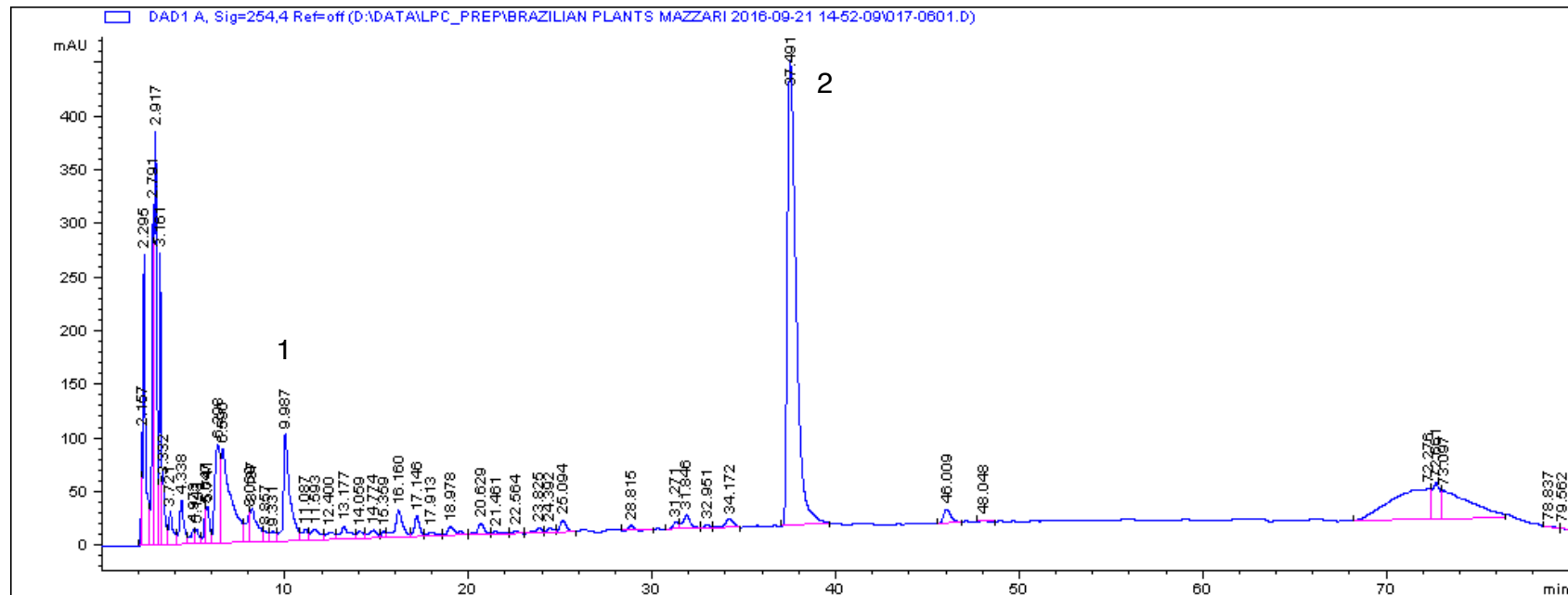
Solanum paniculatum 360nm



5,6 Glycosilated flavonoids

4 Not identified

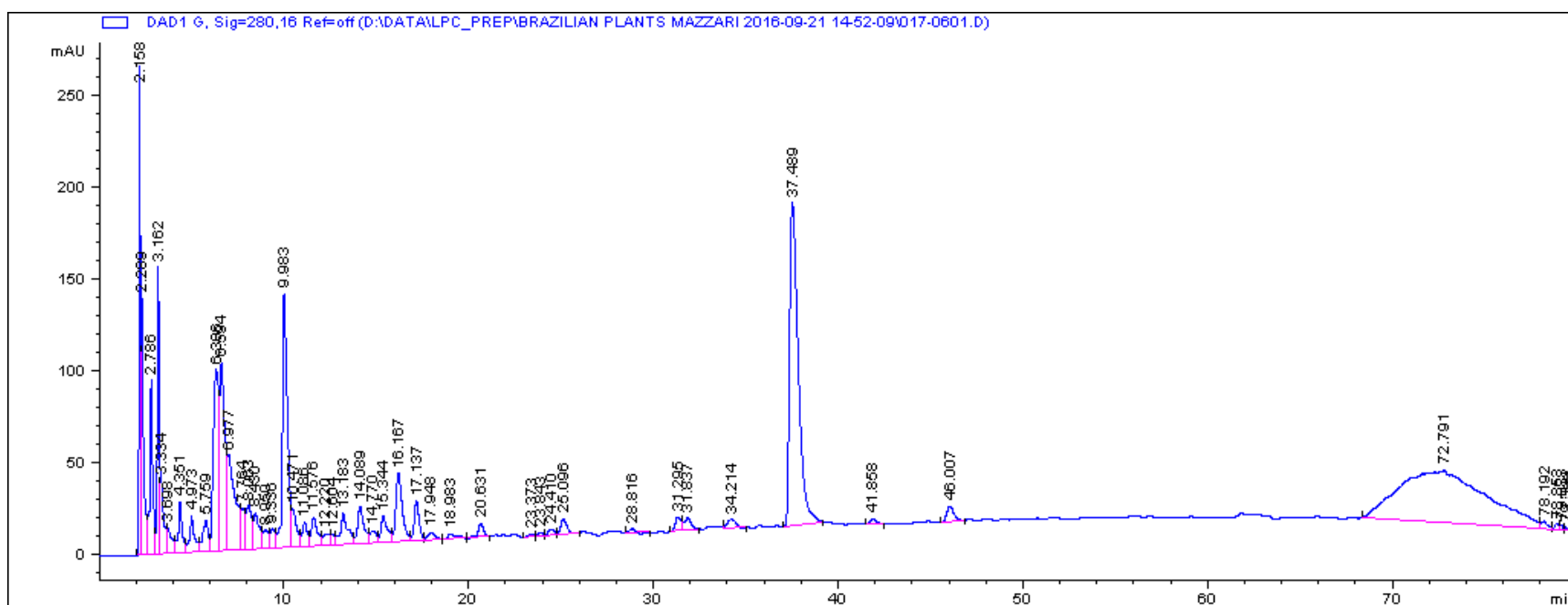
Solidago microglossa 254nm



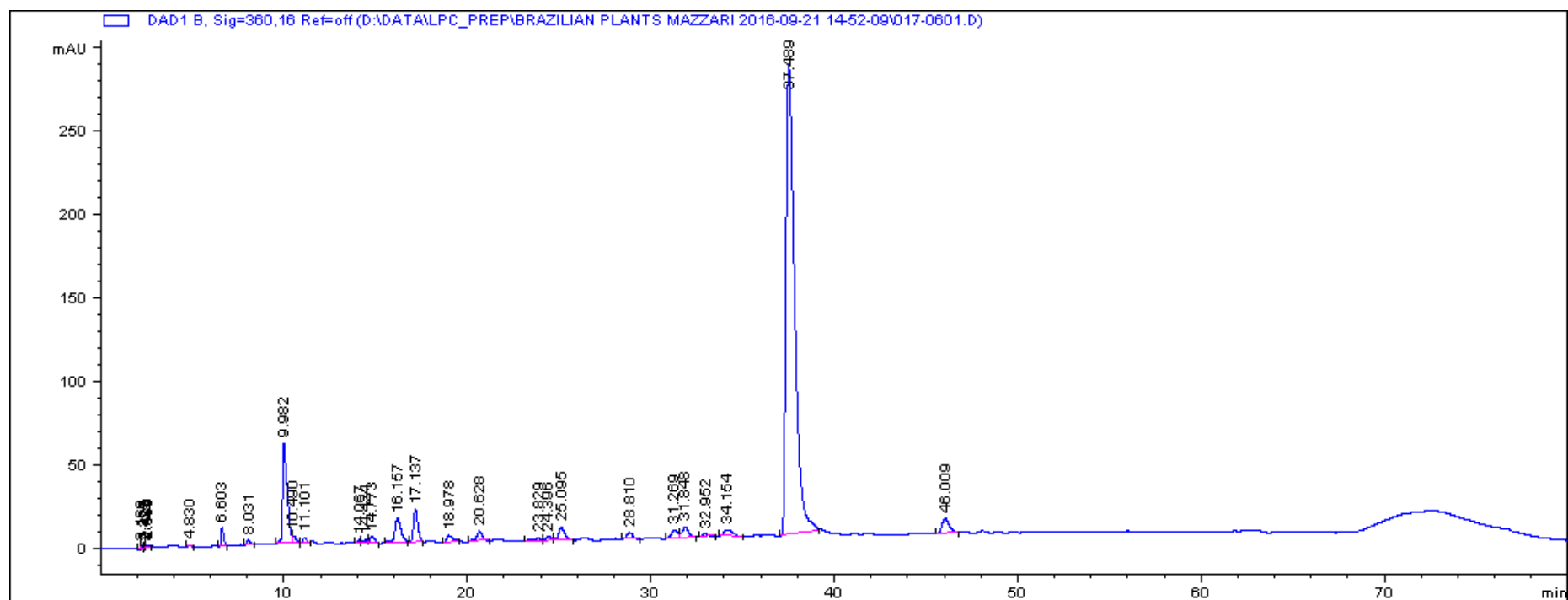
1 Chlorogenic acid

2 Glycosilated
flavonoids

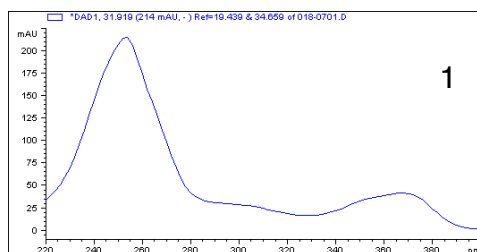
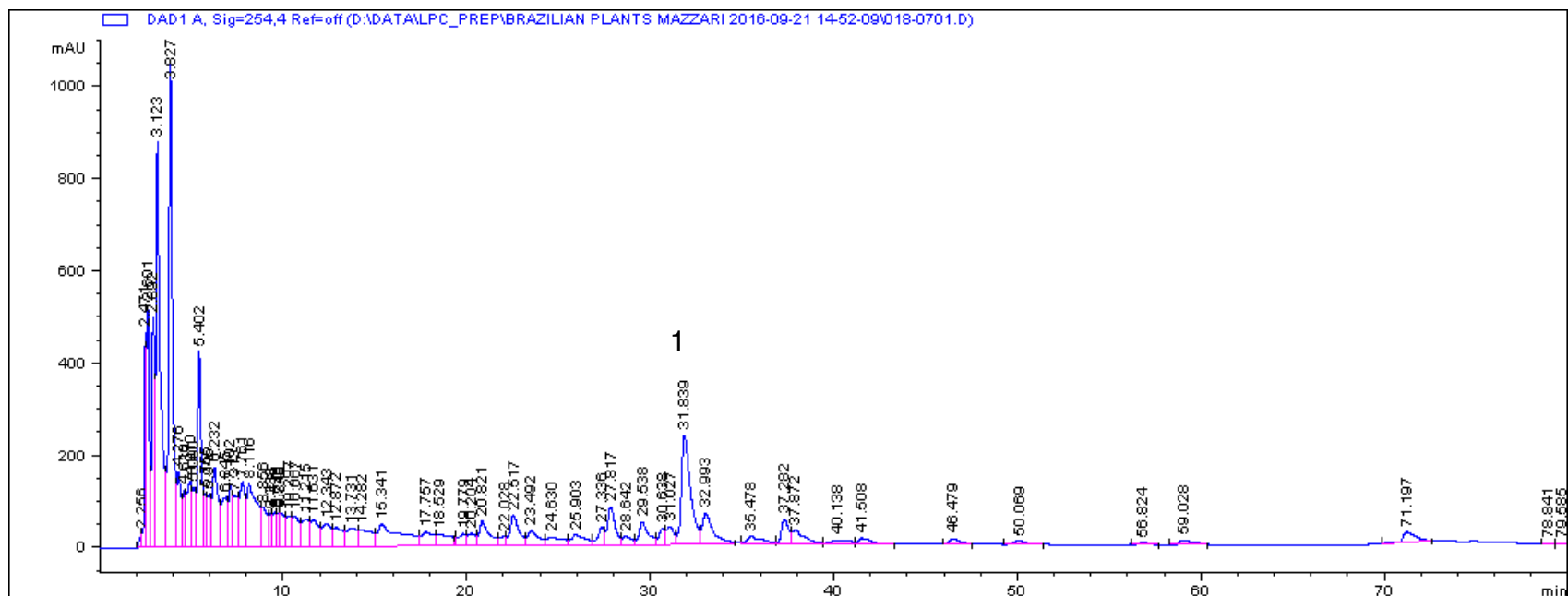
Solidago microglossa 280nm



Solidago microglossa 360nm

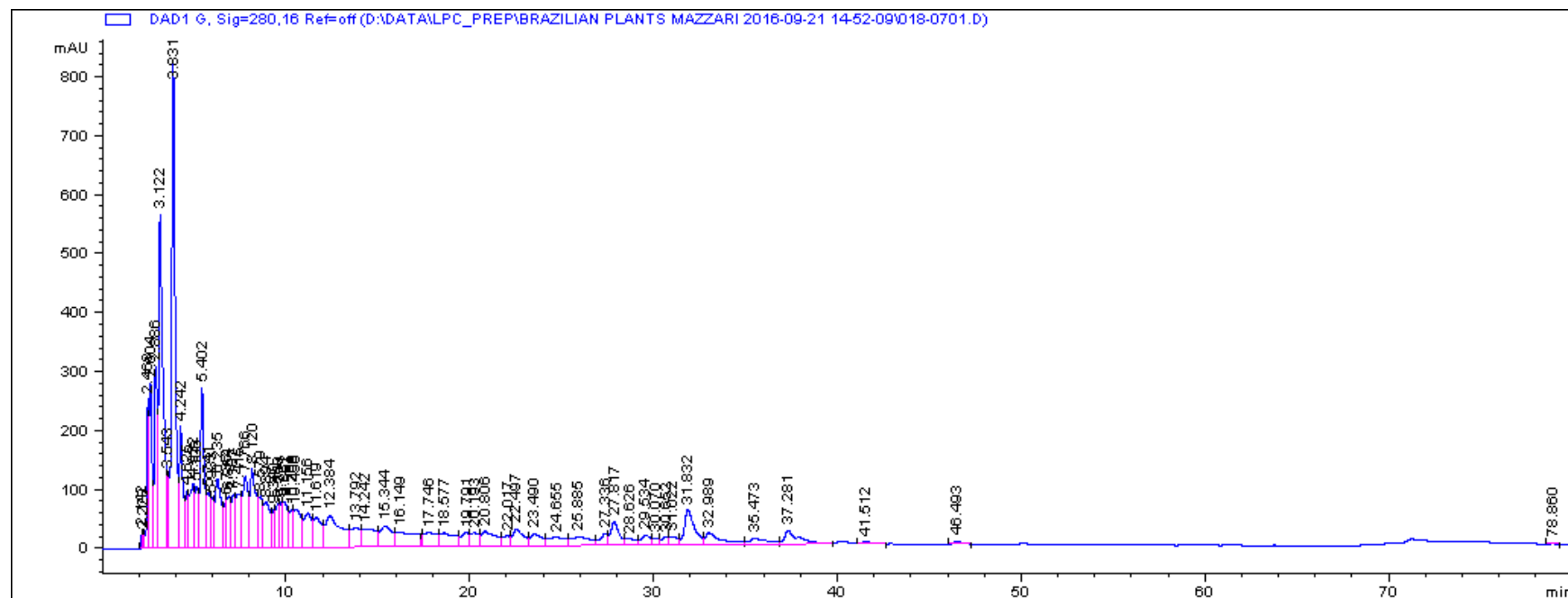


Syzygium jambolanum 254nm

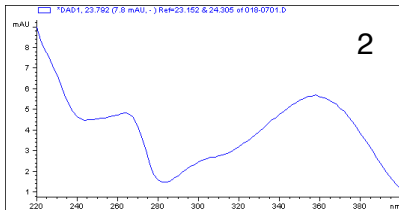
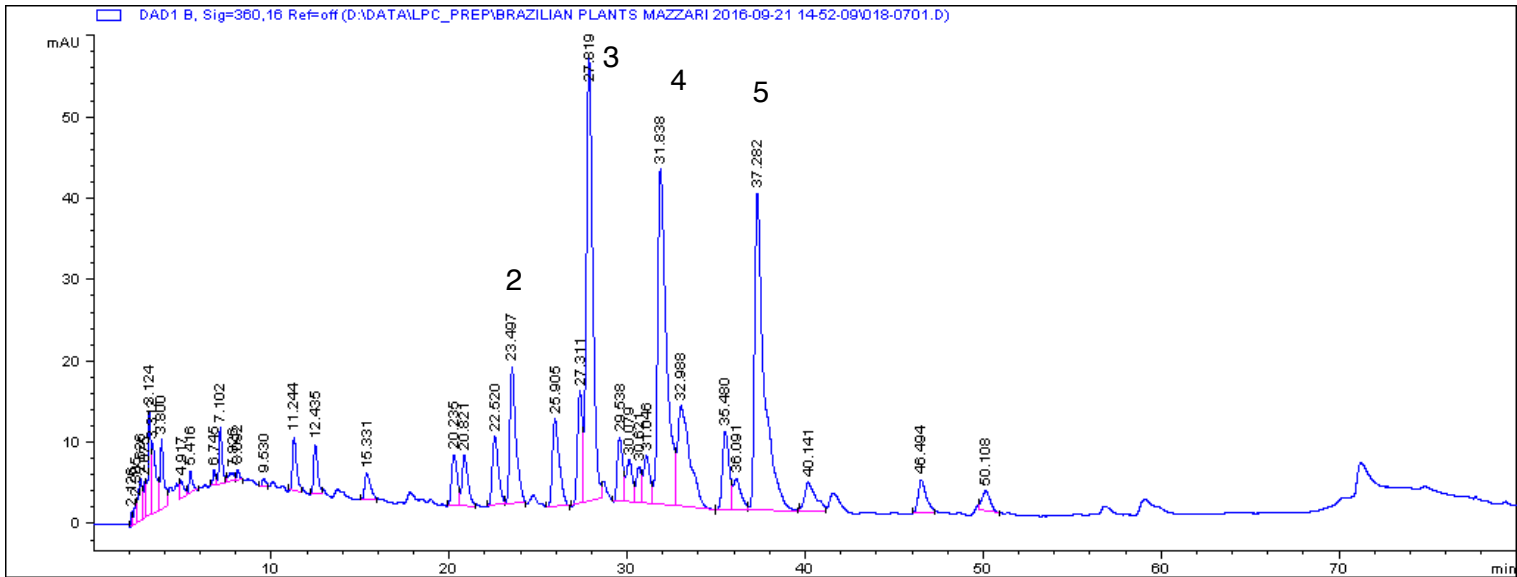


1 Not identified

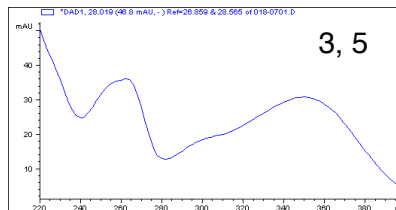
Syzygium jambolanum 280nm



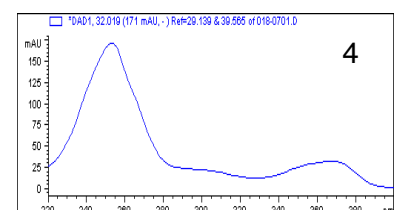
Syzygium jambolanum 360nm



2 Not identified

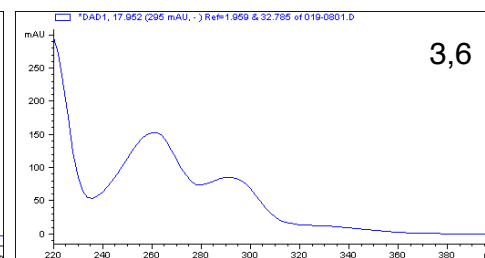
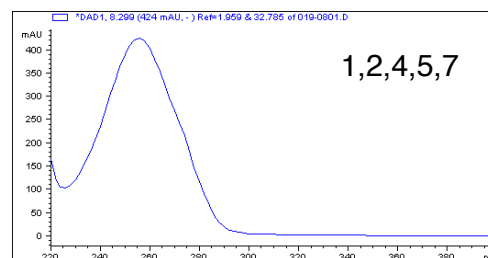
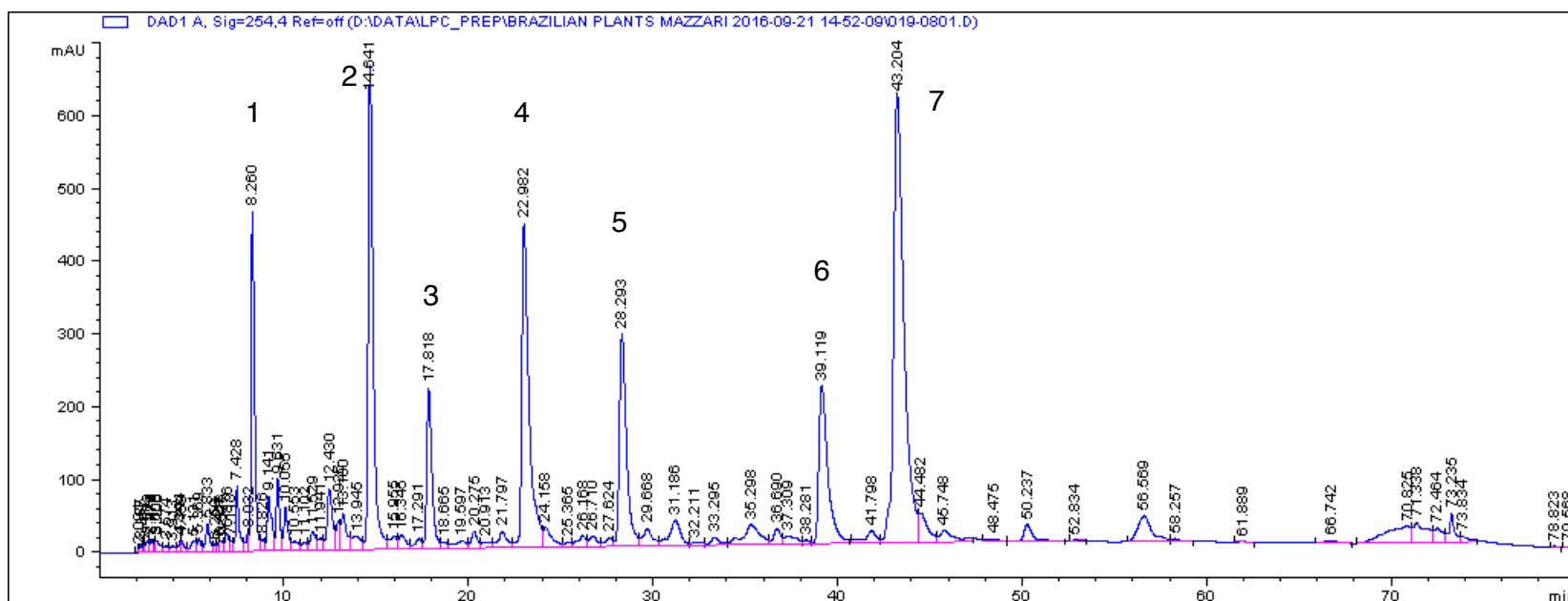


3,5 Glycosilated flavonoids



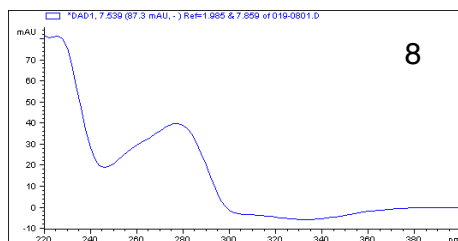
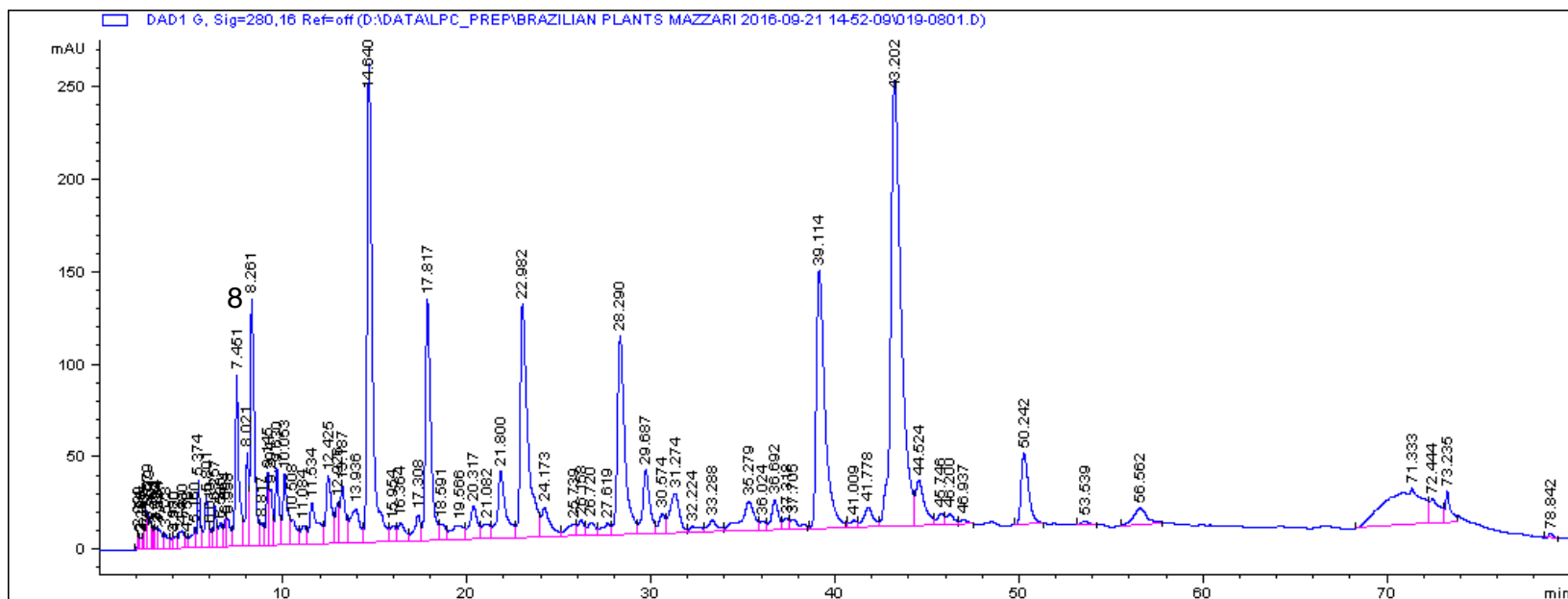
4 Not identified

Tabebuia avellanedae 254nm



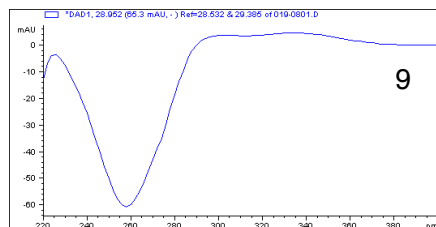
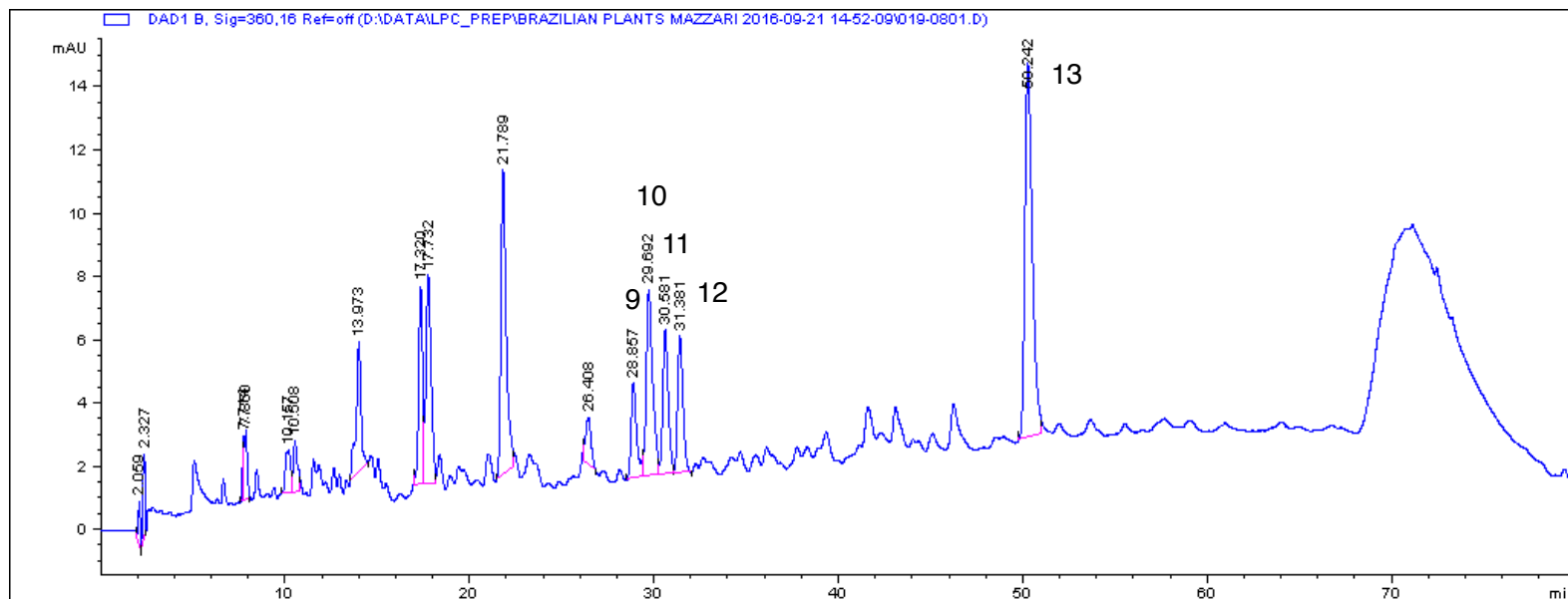
1-7 Not identified

Tabebuia avellanedae 280nm

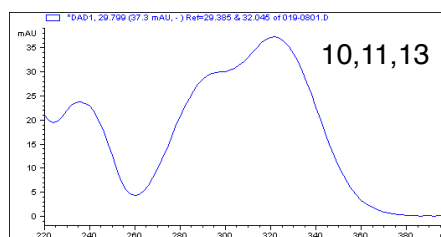


8 Tannin

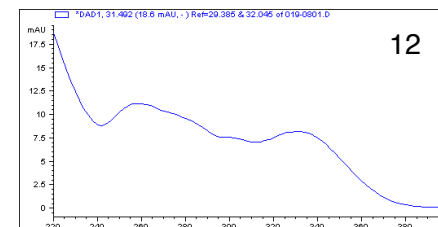
Tabebuia avellanedae 360nm



9 Not identified

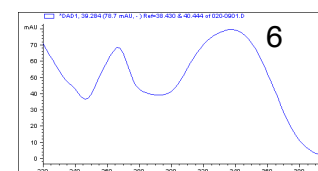
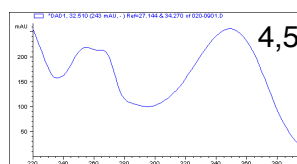
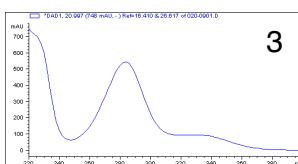
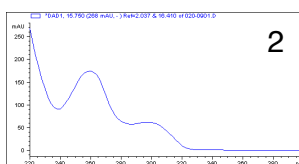
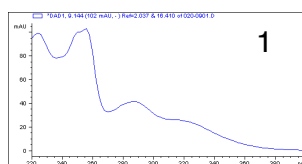
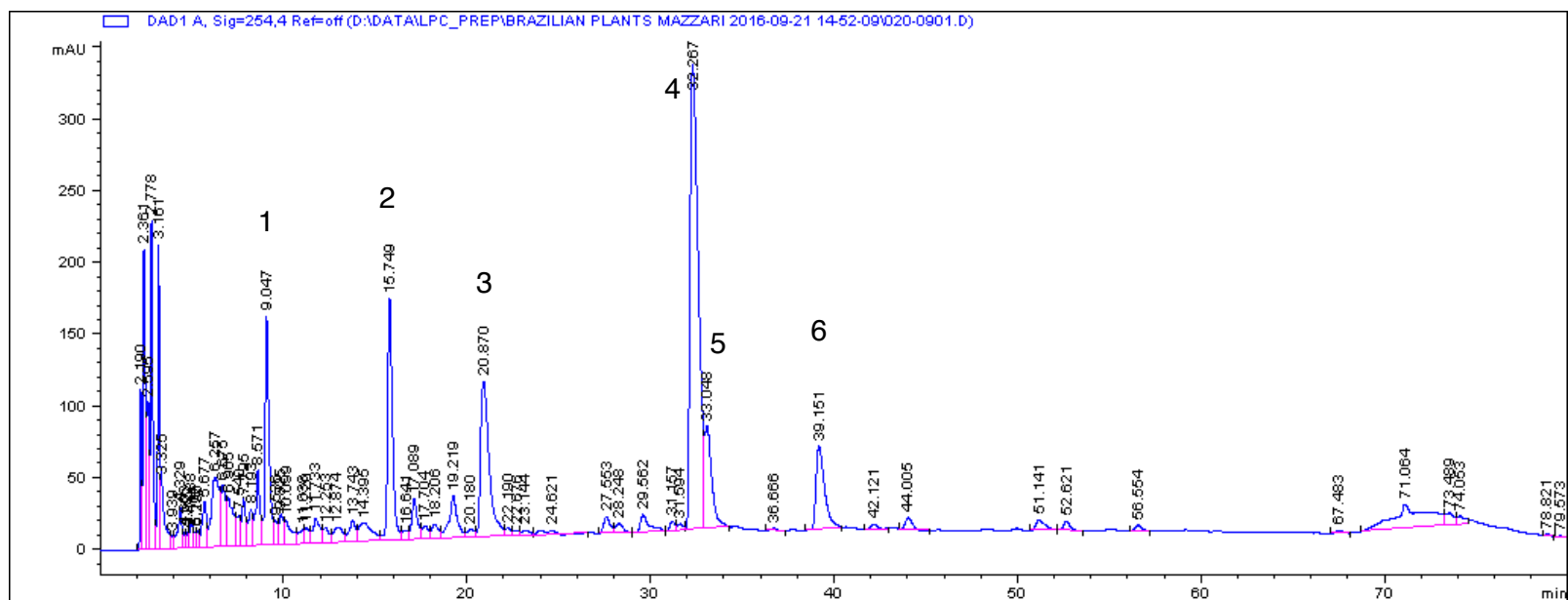


10,11,13 Phenolic acids



12 Not identified

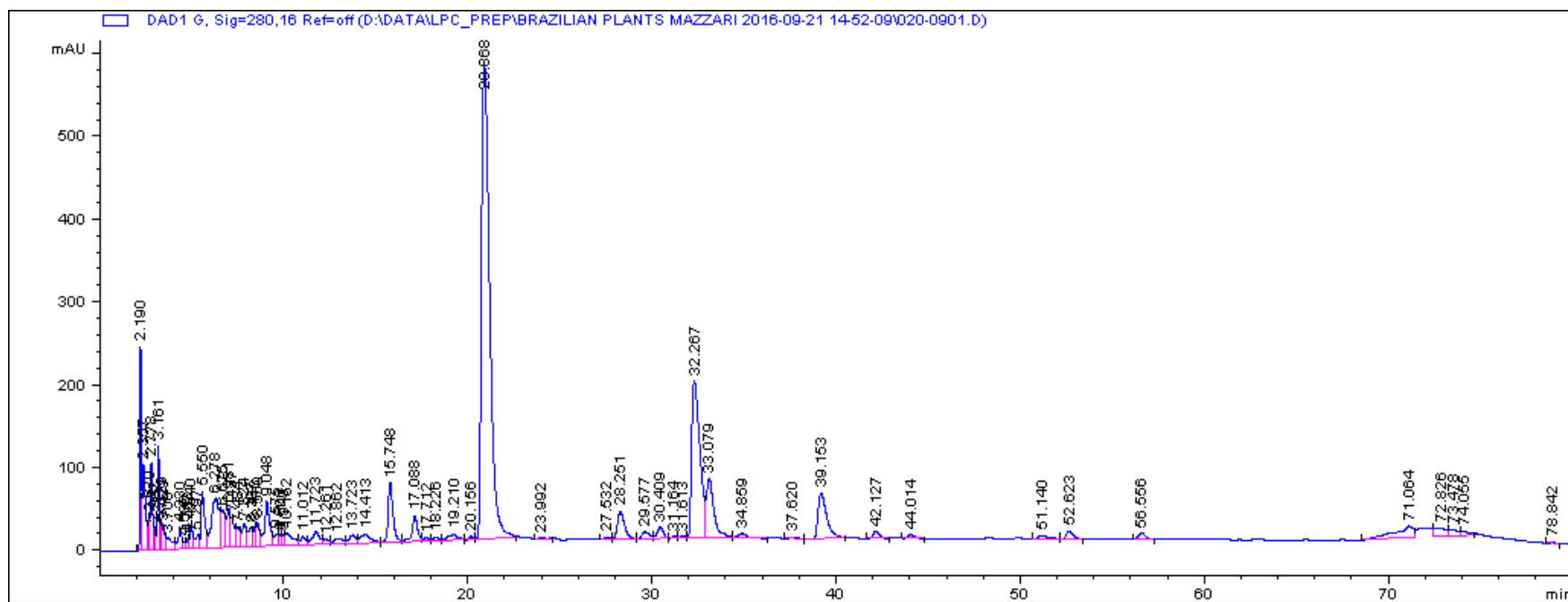
Vernonia polyanthes 254nm



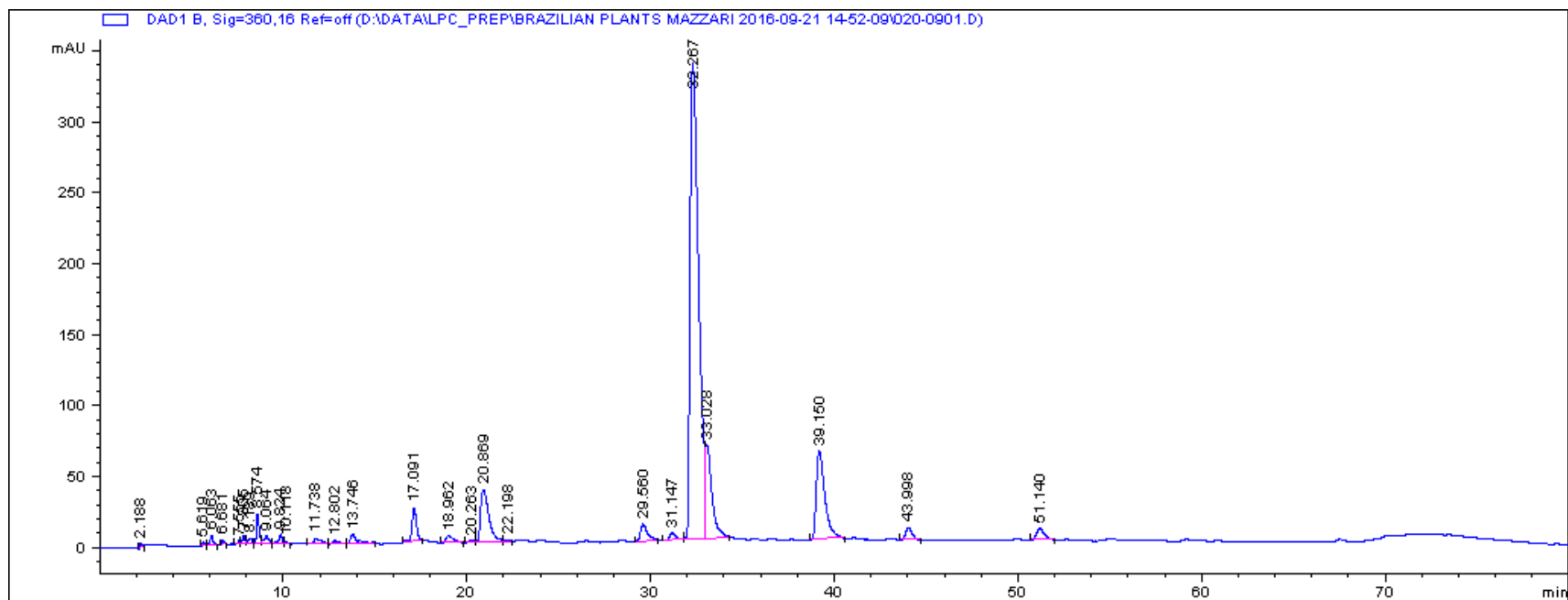
1-3 Not identified

4,5,6 Glycosilated flavonoids

Vernonia polyanthes 280nm



Vernonia polyanthes 360nm



7.6 TABULAR SUMMARY OF CYP3A4 MRNA GENE EXPRESSION IN HEPG2 CELLS (RESULTS SHOW MEAN \pm SEM, N \geq 3)

Samples	Sample ID	Fold induction/inhibition normalised with β -actin
Control (DMSO 0.2%)	CTR	1
RIF 50 μ M	RIF	4.95 \pm 0.26
DMSO 1%	DMS	0.37 \pm 0.05
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	4.24 \pm 0.30
<i>Artemisia absinthium</i> L.	AA1	1.41 \pm 0.24
<i>Bidens pilosa</i> L.	BP1	0.84 \pm 0.18
<i>Casearia silvestris</i> Sw.	CAS	1.57 \pm 0.16
<i>Cordia verbenaceae</i> A. DC.	CV1	1.45 \pm 0.17
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	1.09 \pm 0.21
<i>Equisetum arvense</i> L.	EA1	1.62 \pm 0.27
<i>Erythrina mulungu</i> Benth.	EM1	0.87 \pm 0.10
<i>Lamium album</i> L.	LA1	0.51 \pm 0.15
<i>Lippia sidoides</i> Cham.	LS1	1.44 \pm 0.16
<i>Malva sylvestris</i> L.	MSF	0.75 \pm 0.10
	MSH	0.76 \pm .011
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MI1	1.84 \pm 0.24
<i>Morus nigra</i> L.	MN1	1.39 \pm 0.23
<i>Passiflora incarnata</i> L.	PI1	1.82 \pm 0.21
<i>Persea americana</i> Mill.	PA1	2.02 \pm 0.57
<i>Rhamnus purshiana</i> DC.	RP1	1.53 \pm 0.23
<i>Salix alba</i> L.	SA1	3.25 \pm 0.29
<i>Schinus terebinthifolia</i> Raddi	ST1	4.88 \pm 0.19
<i>Solanum paniculatum</i> L.	SP1	0.41 \pm 0.15
<i>Solidago microglossa</i> DC.	SM1	1.05 \pm 0.06
<i>Syzygium jambolanum</i> (Lam.) DC.	SJ1	4.22 \pm 0.10
<i>Tabebuia avellanedae</i> Lorentz ex Griseb.	TA1	1.32 \pm 0.25
<i>Vernonia polyanthes</i> Less.	VP1	4.22 \pm 0.24

7.7 TABULAR SUMMARY OF INTRACELLULAR GSH

CONCENTRATIONS IN HEPG2 CELLS (RESULTS SHOW MEAN \pm

SEM, N \geq 3)

Samples	Sample ID	GSH (μ M /10 ⁵ cells)
Control (water)	CTR	29.91 \pm 0.36
Buthionine sulfoximine (BSO) 10 μ M	BSO	17.78 \pm 1.70
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	16.32 \pm 1.52
<i>Artemisia absinthium</i> L.	AA1	27.93 \pm 1.72
<i>Bidens pilosa</i> L.	BP1	17.61 \pm 1.79
<i>Casearia silvestris</i> Sw.	CAS	18.77 \pm 1.83
<i>Cordia verbenaceae</i> A. DC.	CV1	15.98 \pm 1.80
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	22.23 \pm 0.76
<i>Equisetum arvense</i> L.	EA1	21.76 \pm 2.01
<i>Erythrina mulungu</i> Benth.	EM1	47.05 \pm 2.0
<i>Lamium album</i> L.	LA1	15.98 \pm 1.28
<i>Lippia sidoides</i> Cham.	LS1	26.34 \pm 1.20
<i>Malva sylvestris</i> L.	MSF	25.94 \pm 1.28
	MSH	17.96 \pm 1.72
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MI1	8.55 \pm 1.32
<i>Morus nigra</i> L.	MN1	31.54 \pm 1.50
<i>Passiflora incarnata</i> L.	PI1	16.82 \pm 0.70
<i>Persea americana</i> Mill.	PA1	9.33 \pm 0.57
<i>Rhamnus purshiana</i> DC.	RP1	11.54 \pm 0.50
<i>Salix alba</i> L.	SA1	22.37 \pm 2.51
<i>Schinus terebinthifolia</i> Raddi	ST1	15.70 \pm 1.52
<i>Solanum paniculatum</i> L.	SP1	16.83 \pm 1.95
<i>Solidago microglossa</i> DC.	SM1	21.87 \pm 2.25
<i>Syzygium jambolanum</i> (Lam.) DC.	SJ1	27.35 \pm 1.52
<i>Tabebuia avellanedae</i> Lorentz ex Griseb.	TA1	11.23 \pm 1.60
<i>Vernonia polyanthes</i> Less.	VP1	19.96 \pm 1.81

7.8 TABULAR SUMMARY OF GGT ACTIVITY IN TERMS OF μM P-NITROANILINE CONCENTRATION IN HEPG2 CELLS (RESULTS SHOW MEAN \pm SEM, N \geq 3)

Samples	Sample ID	μM p-nitroaniline per 10^6 cells
Control (water)	CTR	82.30 \pm 7.48
Acivicin 5 μM	ACV	25.45 \pm 7.18
<i>Artemisia absinthium</i> L.	AA1	94.05 \pm 10.95
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	134.65 \pm 6.26
<i>Bidens pilosa</i> L.	BP1	76.93 \pm 3.33
<i>Casearia sylvestris</i> Sw.	CAS	75.99 \pm 6.79
<i>Cordia verbenaceae</i> A. DC.	CV1	43.56 \pm 7.26
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	52.50 \pm 12.05
<i>Equisetum arvense</i> L.	EA1	79.34 \pm 8.72
<i>Erythrina mulungu</i> Benth.	EM1	76.47 \pm 7.10
<i>Lamium album</i> L.	LA1	79.87 \pm 5.92
<i>Lippia sidoides</i> Cham.	LS1	101.46 \pm 10.15
<i>Malva sylvestris</i> L.	MSF	89.01 \pm 9.89
	MSH	84.45 \pm 9.48
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MI1	66.61 \pm 7.19
<i>Morus nigra</i> L.	MN1	84.43 \pm 2.41
<i>Passiflora incarnata</i> L.	PI1	109.82 \pm 9.22
<i>Persea americana</i> Mill.	PA1	56.64 \pm 4.26
<i>Rhamnus purshiana</i> DC.	RP1	131.80 \pm 5.64
<i>Salix alba</i> L.	SA1	52.93 \pm 4.12
<i>Schinus terebinthifolia</i> Raddi	ST1	54.70 \pm 7.58
<i>Solanum paniculatum</i> L.	SP1	83.87 \pm 8.24
<i>Solidago microglossa</i> DC.	SM1	86.01 \pm 6.29
<i>Syzygium jambolanum</i> (Lam.) DC.	SJ1	53.19 \pm 5.10
<i>Tabebuia avellaneda</i> Lorentz ex Griseb.	TA1	73.50 \pm 13.01
<i>Vernonia polyanthes</i> Less.	VP1	78.89 \pm 1.81

7.9 EFFECT OF PLANT EXTRACTS ON INTRACELLULAR RH-123

ACCUMULATION IN CACO-2 VCR CELLS (RESULTS SHOW MEAN

± SEM, N ≥ 3)

Samples	Sample ID	Intracellular Rh-123 Fluorescence (% of control)
Control (Rh-123)	CTR	100 ± 1.50
Verapamil 20µM	VPM	181.78 ± 12.97
<i>Apuleia ferrea</i> (Mart.) Baill.	AF1	412.72 ± 63.72
<i>Artemisia absinthium</i> L.	AA1	113.61 ± 21.77
<i>Bidens pilosa</i> L.	BP1	73.59 ± 25.75
<i>Casearia sylvestris</i> Sw.	CAS	79.28 ± 31.73
<i>Cordia verbenaceae</i> A. DC.	CV1	108.45 ± 52.15
<i>Costus spicatus</i> (Jacq.) Sw.	CS1	81.98 ± 21.22
<i>Equisetum arvense</i> L.	EA1	108.36 ± 32.48
<i>Erythrina mulungu</i> Benth.	EM1	69.64 ± 24.72
<i>Lamium album</i> L.	LA1	106.18 ± 7.03
<i>Lippia sidoides</i> Cham.	LS1	104.63 ± 36.46
<i>Malva sylvestris</i> L.	MSF	100.06 ± 19.39
<i>Maytenus ilicifolia</i> Mart. ex Reissek	MSH	97.61 ± 11.02
<i>Morus nigra</i> L.	MI1	96.79 ± 17.03
<i>Passiflora incarnata</i> L.	MN1	68.12 ± 23.14
<i>Persea americana</i> Mill.	PI1	108.59 ± 18.28
<i>Rhamnus purshiana</i> DC.	PA1	90.61 ± 25.40
<i>Salix alba</i> L.	RP1	78.50 ± 21.47
<i>Schinus terebinthifolia</i> Raddi	SA1	192.25 ± 16.37
<i>Solanum paniculatum</i> L.	ST1	442.61 ± 48.55
<i>Solidago microglossa</i> DC.	SP1	87.14 ± 34.63
<i>Syzygium jambolanum</i> (Lam.) DC.	SM1	105.70 ± 41.44
<i>Tabebuia avellaneda</i> Lorentz ex Griseb.	SJ1	182.39 ± 14.82
<i>Vernonia polyanthes</i> Less.	TA1	87.36 ± 7.20
	VP1	101.61 ± 2.70

7.10 PUBLICATIONS

7.10.1 Herbal medicines in Brazil: pharmacokinetic profile and potential herb-drug interactions



Herbal medicines in Brazil: pharmacokinetic profile and potential herb-drug interactions

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A plethora of active compounds found in herbal medicines can serve as substrate for enzymes involved in the metabolism of xenobiotics. When a medicinal plant is co-administered with a conventional drug and little or no information is known about the pharmacokinetics of the plant metabolites, there is an increased risk of potential herb-drug interactions. Moreover, genetic polymorphisms in a population may act to predispose individuals to adverse reactions. The use of herbal medicines is rapidly increasing in many countries, particularly Brazil where the vast biodiversity is a potential source of new and more affordable treatments for numerous conditions. Accordingly, the Brazilian Unified Public Health System (SUS) produced a list of 71 plant species of interest, which could be made available to the population in the near future. Physicians at SUS prescribe a number of essential drugs and should herbal medicines be added to this system the chance of herb-drug interactions further increases. A review of the effects of these medicinal plants on Phase 1 and Phase 2 metabolic mechanisms and the transporter P-glycoprotein was conducted. The results have shown that approximately half of these medicinal plants lack any pharmacokinetic data. Moreover, most of the studies carried out are *in vitro*. Only a few reports on herb-drug interactions with essential drugs prescribed by SUS were found, suggesting that very little attention is being given to the safety of herbal medicines. Here we have taken this information to discuss the potential interactions between herbal medicines and essential drugs prescribed to Brazilian patients whilst taking into account the most common polymorphisms present in the Brazilian population. A number of theoretical interactions are pinpointed but more pharmacokinetic studies and pharmacovigilance data are needed to ascertain their clinical significance.

Keywords: herb-drug interactions, cytochrome P450, glutathione, glucuronidation, P-glycoprotein, polymorphism, Brazil, pharmacovigilance

INTRODUCTION

Consumption of herbal medicines has been increasing worldwide over the past few years. In developed countries, such as the United Kingdom, around 50% of the population have used herbal medicines at least once in their life and, surprisingly, almost 100% of HIV patients in the country have admitted to using herbal medicines (Caminal Homar, 2005). In developing countries, the World Health Organization (WHO) estimates that 65–80% of the population relies on herbal medicines as a primary source of treatment (Rahman and Singhal, 2002). These statistics are in line with Brazil, where 66% of the population have no access to commercial medicines (Trojan-Rodrigues et al., 2012). Even when access is granted, popular use of herbal medicines is often due to poor medical and pharmaceutical assistance and the high cost of treatment with conventional drugs (Silveira et al., 2008). Brazilians are also becoming more interested in “safe” and “natural” treatments aimed to promote healthier living. A consequence of this increased use of phytotherapy has been a higher number of case reports on adverse reactions caused by uncontrolled consumption of herbal medicines (Silveira et al., 2008).

The Brazilian Health Surveillance Agency (ANVISA) is in charge of the regulation of herbal medicines in the country and since its creation in 1999, many advances have been made in order to control and ensure their efficacy and safety (Balbino and Dias, 2010). To guarantee that the phytomedicinal candidate is safe for consumption by humans, the Agency established a requirement that at least 20 years of prior traditional use must be attested. In the absence of this evidence the efficacy and safety of the candidate needs to be demonstrated by a point based system according to literature data, preclinical and clinical tests or indication that the herb is already included in the List of Simplified Registration of Herbal Medicines (ANVISA, 2010). Although these regulations are relevant to improve the safety and efficacy of herbal medicines, pharmacokinetic studies on the plants are not yet a regulatory requirement. As a consequence, there is a scarcity of this kind of data which is regarded as an oversight in terms of safety (Ribeiro et al., 2005).

The SUS is one of the biggest public health systems in the world, responsible for approximately 140 million Brazilian citizens (Mendes, 2013). In order to establish which medicines

should be provided by SUS, the Brazilian Health Ministry produced a list of essential drugs according to the International Classification of Diseases (ICD) and epidemiological studies conducted nationwide (Saúde, 2010; SUS, 2010). Several herbal medicines are already part of this list, such as *Cynara scolymus* L., *Schinus terebinthifolius*, and *Rhamnus purshiana*. However, due to the extensive number of plant species possessing pharmacological activity used in Brazil, the Health Ministry determined that more herbal medicines should be provided by the System to the population, as part of one of the aims of the National Policy of Integrative and Complementary Practices (PNPIC). Consequently, the list of medicinal plants of interest of SUS (RENISUS) was published in 2008 and, from this point on, efforts have been concentrated on elucidating the efficacy and safety of the 71 plant species present on the list (Saúde, 2009; SUS, 2009; cf. Feijó et al., 2012).

The pharmacokinetic profile of pharmaceutical drugs is essential to determine whether or not they will interact with other therapeutic interventions (Ionescu and Caira, 2005). Polymorphism studies are also relevant because they may impact the capacity to metabolize xenobiotics, thus leading to adverse drug reactions (ADRs) within certain ethnicities (Suarez-Kurtz, 2005). However, pharmacokinetic studies on medicinal plants are very difficult to carry out because of their chemical complexity (Simões and Mariot, 2003). As a result, there is very little data for numerous native and exotic plants that are traditionally used in Brazil (He et al., 2010). The *in vitro* pharmacokinetic profile of a few herbal medicines can be found in the literature and, although these data are important, they are seldom used for the prediction of potential herb-drug interactions. Thus, the aim of this article is to provide an overview and critical evaluation of the pharmacokinetic data of medicinal plants to be used in the Brazilian health system. By discussing the potential herb-drug interactions with essential drugs upon Phase 1 and Phase 2 metabolic mechanisms and P-Glycoprotein activity, we intend to prompt race awareness on the safety of herbal medicines in Brazil.

METHODOLOGY

A literature search was conducted using the Library of Medicine's PubMed database between May and June 2013. Included studies consisted of the reported effects of the medicinal plants of RENISUS list on the main liver metabolic enzymes, which are involved in Phase 1 (functionalization reactions mediated by cytochrome P450) and Phase 2 (glutathione conjugation, glucuronidation, sulfation, methylation and acetylation) metabolism and also the assessment of their effects on P-glycoprotein activity. The following combinations of keywords were used: "Plant name AND, 1A2, 3A4, 3A5, 3A7, 2C9, 2C19, 2D6, 2E1," "Plant name AND glutathione," or "Plant name AND gsh," "Plant name AND glucuronidation" or "Plant name AND ugt," "Plant name AND sulfation," "Plant name AND sulfate conjugation," "Plant name AND sulfotransferase," "Plant name AND methylation," "Plant name AND methyltransferase," "Plant name AND acetylation," "Plant name AND n-acetyltransferase," "Plant name AND P-glycoprotein" or "Plant name AND Pgp." EndNote web was the citation tool used to manage and organize all the references collected.

It is important to note that the list of Brazilian medicinal plants of interest of SUS encompass native and exotic adapted plant species and that we are aware of the chemical variability of the materials, which eventually may be harmonized by pharmacopoeial monographs of their respective countries.

PHASE 1 METABOLISM AND THE HUMAN LIVER CYTOCHROMES P450

Xenobiotic metabolism is normally divided into two phases: Phase 1 (functionalization reactions) and Phase 2 (conjugative reactions). Phase 1 reactions prepare the drug for Phase 2 metabolism by adding polar functional groups to the xenobiotic (Ionescu and Caira, 2005).

Human drug-metabolizing enzymes are present ubiquitously in the body. Over 50 human cytochromes P450 have already been isolated; the major ones found in the liver include CYP1A2, CYP2E1, CYP2C9/19, CYP2D6, and CYP3A4/5/7 (Figure 1) (Gibson and Skett, 2001). The CYP 1, 2, and 3 are the most abundant families of CYP metabolizing enzymes and the CYP1A2, CYP2C, and CYP3A4 isoforms account for about 30% of the metabolism of the majority of drugs (Atkinson, 2012).

The cytochrome P450 monooxygenase enzymes are located in the smooth endoplasmic reticulum of the liver and other extra hepatic tissues. Numerous drugs are metabolized by cytochrome P450 enzymes through oxidation reactions such as aromatic and aliphatic hydroxylation, epoxidation, *N*-dealkylation, *O*-dealkylation, *S*-dealkylation, oxidative deamination, *N*-oxidation, *S*-oxidation, phosphothionate oxidation, dehalogenation, and alcohol oxidation. Reduction, hydrolysis and hydration are other examples of Phase 1 reactions catalyzed by the cytochrome P450 (Ionescu and Caira, 2005).

Similar to conventional drugs, herbal medicines also undergo Phase 1 and Phase 2 metabolism in order to be excreted from the body. If a herbal medicine is concomitantly used with a conventional drug, for example, the first may inhibit or induce the activity and expression of a specific cytochrome that could be the same one responsible for the metabolism of the latter, leading to herb-drug interactions (Hu et al., 2005).

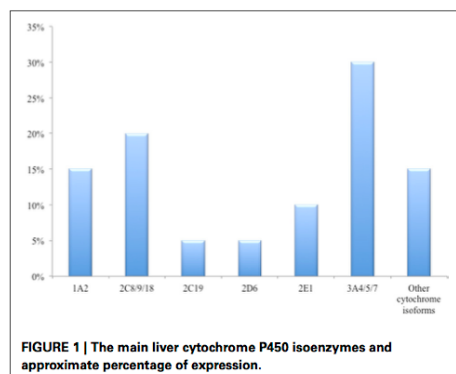


FIGURE 1 | The main liver cytochrome P450 isoenzymes and approximate percentage of expression.

GENETIC POLYMORPHISM

Responses to drug consumption differ among individuals due to the variability of CYP content. A predefined dosage of a medicine might be enough to exert a pharmacological effect in one patient but it may be necessary to be adjusted for another patient to achieve the same effect. This can be explained by genetic polymorphisms within CYPs that can affect the metabolism of xenobiotics in general, leading to changes in drug response and increased risk of ADRs (Zhou et al., 2009). For example, the bioavailability of Omeprazole, which is a drug that is metabolized by CYP3A4 and CYP 2C19, demonstrated to be slower in Mexicans when compared with Caucasians but faster than that of Asians. The ethnic mixture of the Mexican population is a plausible explanation for differences in metabolic response compared to other ethnic groups (Gonzalez et al., 2003).

The genomic diversity of the Brazilian population is a result of the genetic admixture of three groups: Europeans, Africans and Amerindians. Due to this very distinctive miscegenation, polymorphisms in cytochromes levels are undoubtedly present among Brazilians (Suarez-Kurtz, 2005). The Brazilian National Pharmacogenetics/Pharmacogenomics Network (REFARGEN) carried out studies in four regions of Brazil in order to map the genetic diversity of the population (Suarez-Kurtz, 2004). The study divided the population into three distinct races: white, brown and black. The results obtained from REFARGEN showed that numerous polymorphisms were found on cytochromes 2C9, 2C19, 2D6, and 3A5.

Taking all these points into consideration, from the data gathered in our literature review we will summarize some potentially clinically relevant pharmacokinetic effects of herbal medicines in metabolic enzymes responsible for Phase 1 and Phase 2 metabolisms and P-glycoprotein activity. Also, the presence of polymorphisms among the Brazilian population and its implication in the metabolism were assessed. Some interactions between essential drugs and herbal medicines in Brazil found in the literature will be shown, which will eventually need to be put to test in the laboratory or followed up epidemiologically.

EFFECTS OF HERBAL MEDICINES IN PHASE 1 ENZYMES AND HERB-DRUG INTERACTIONS

CYP1A2

This cytochrome isoform is mainly found in the liver (15%) and it metabolizes almost 20% of the current therapeutic arsenal (Wang and Zhou, 2009). Amid the 71 plant species, nine of them were found to interfere with the activity of CYP1A2 (Table 1). Examples of essential drugs, which are substrates for the CYP1A2, include the highly popular painkiller Acetaminophen, the beta-blocker Propranolol, the antidepressant Clomipramine and the anticoagulant Warfarin.

Acetaminophen (Paracetamol) is bioactivated via CYP1A2, CYP3A4, and CYP2E1, resulting in the production of the toxic compound N-acetyl-*p*-benzoquinone (NAPQI). In normal conditions, the drug is detoxified by conjugation with glutathione. However, in cases of overdose, this metabolic route becomes saturated, increasing the bioactivation of NAPQI and its binding to other molecules, such as proteins, resulting in damages to the liver due to cell death (Lee et al., 2001). Intake of Acetaminophen

Table 1 | Medicinal plant species listed in RENISUS with reported effects of on CYP1A2.

Plant species/Family	Effects on CYP1A2	References
<i>Allium sativum</i> (Aliaceae)	+	Le Bon et al., 2003
<i>Curcuma longa</i> (Zingiberaceae)	+	Thapliyal et al., 2002
<i>Eucalyptus globulus</i> (Myrtaceae)	–	Unger and Frank, 2004
<i>Glycine max</i> (Leguminosae)	–	Shon and Nam, 2004
<i>Harpagophytum procumbens</i> (Pedaliaceae)	NE, –	Unger and Frank, 2004; Modarai et al., 2011
<i>Mentha piperita</i> (Lamiaceae)	–	Unger and Frank, 2004
<i>Phyllanthus amarus</i> (Euphorciaceae)	–	Hari Kumar and Kuttan, 2006
<i>Punica granatum</i> (Lythraceae)	–	Faria et al., 2007a
<i>Trifolium pratense</i> (Fabaceae)	–	Unger and Frank, 2004

+, Enzyme induction; –, Enzyme inhibition; NE, No Effect.

with *Allium sativum* and *Curcuma longa* could theoretically increase the toxicity of the drug due to accumulation of NAPQI caused by induction of CYP1A2. On the other hand, consumption of Acetaminophen with medicinal plants such as *Phyllanthus amarus*, *Mormodica charantia*, *Eucalyptus globulus*, *Glycine max*, *Harpagophytum procumbens*, *Mentha piperita*, *Trifolium pratense* and *Punica granatum* based remedies may decrease levels of this toxic metabolite because of CYP1A2 enzyme inhibition.

Although it appears that garlic consumption can put the integrity of hepatocytes at risk, a study conducted by Gwilt et al. (1994) proved that the garlic extract administration has little effect on the metabolism of Acetaminophen. In this study 16 male nonsmokers took 10 ml of garlic extract daily for 3 months. Acetaminophen was administered at five different time points: before garlic treatment, at the end of the first, second, and third month of garlic extract consumption and 1 month after interruption of garlic treatment. The results demonstrated that garlic extract does not interfere with the oxidative pathway of Acetaminophen and therefore it does not represent a potential risk for hepatocytes.

The beta-blocker Propranolol and the tricyclic antidepressant Clomipramine are essential drugs extensively prescribed in Brazil. The biotransformation of Propranolol and Clomipramine starts with the *N*-dealkylation, governed by *S*-mephenytoin (CYP1A2) for the former and the *N*-demethylation of the side chain of the molecule of the latter to form the active metabolite desmethylclomipramine (DCIP) (Nielsen et al., 1996). Therefore, plant species that inhibit CYP1A2 must be avoided during the

treatment period with these drugs. This is, however, theoretical and in this case no reports have so far been found in the literature.

The anticoagulant Warfarin is indicated for the treatment of venous thrombosis and pulmonary embolism. The pharmacokinetics of this drug can easily be altered by CYP1A2 inhibitors. A rare case of interaction of warfarin with garlic supplements has been reported, leading to excessive bleeding (Baxter and Stockley, 2008). Other cases of herb-drug interactions were detected after co-administration of *Curcuma longa*, *Glycine max*, *Harpagophytum procumbens*, *Punica granatum*, and *Trifolium pratense* with Warfarin, resulting in pharmacokinetic alterations (Heck et al., 2000; Cambria-Kiely, 2002; Ramsay et al., 2005; Komperda, 2009; Liu et al., 2013).

CYP1A2 is considered to be highly inducible and polymorphic. Two polymorphisms were found to be common in South Brazilians: the CYP1A2*1F and *1K alleles. They cause higher and decreased enzyme expression, respectively (Kohlrausch et al., 2014). Their high frequency in Southern Brazilians suggests that chances of ADRs may be increased within this group.

CYP2C9

The CYP2C9 is the major CYP2C isoform found in the human liver and typical substrates are molecules that contain an anionic site and a hydrophobic site (Mo et al., 2009). This CYP enzyme metabolizes approximately 15% of clinical drugs, including the nonsteroidal anti-inflammatory (NSAID) Ibuprofen, the antihypertensive Losartan, the antidepressant Fluoxetine, the antiepileptic Phenytoin and the anti-hypercholesterolemic Fluvastatin (Baxter and Stockley, 2008; Berka et al., 2011). Certain endogenous bioactive substances such as steroids, melatonin, retinoids and arachidonic acid are also metabolized by this CYP family (Mo et al., 2009).

Ibuprofen is a common medication prescribed by SUS to combat pain and inflammation. The drug is also included in the WHO Model List of Essential Medicines as a drug for pain and palliative care and as an antimigraine medicine (Medicines, 2012; WHO, 2012, 2013). As mentioned above, Ibuprofen is mostly metabolized by CYP2C9 leading to the formation of the active metabolite (S)-ibuprofen. Herbal species that are substrates for CYP2C9 (Table 2), may inhibit the formation of (S)-ibuprofen, potentially leading to therapeutic failure (Mo et al., 2009).

The active metabolites E-3174, norfluoxetine, 4'-HPPH and 6-hydroxy fluvastatin, are formed through the action of CYP2C9 on the essential drugs Losartan, Fluoxetine, Phenytoin and Fluvastatin, respectively (von Moltke et al., 1997; Scripture and Pieper, 2001; Joy et al., 2009). Concomitant consumption of those drugs along with any of the plant species listed on Table 2 could impact on the formation of these substances.

The CYP2C9*2 and *11 alleles were found in higher frequency in white Brazilians, whereas CYP2C9*3 was mostly found in browns and CYP2C9*5 in the black population. These polymorphisms decrease the enzyme activity *in vitro* (Zhou et al., 2009) therefore consumption of medicinal plants such as garlic, eucalyptus, devil's claw, mentha, pomegranate, red clover and ginger may further inhibit their function or expression, potentially causing herb-drug interactions in these ethnic groups.

Table 2 | Medicinal plant species listed in RENISUS with reported effects of on CYP2C9.

Plant species/Family	Effects on CYP2C9	References
<i>Allium sativum</i> (Aliaceae)	–, +	Foster et al., 2001; Ho et al., 2010
<i>Eucalyptus globulus</i> (Myrtaceae)	–	Unger and Frank, 2004
<i>Harpagophytum procumbens</i> (Pedaliaceae)	NE, –	Modarai et al., 2011
<i>Mentha piperita</i> (Lamiaceae)	–	Unger and Frank, 2004
<i>Punica granatum</i> (Lythraceae)	–	Hanley et al., 2012
<i>Trifolium pratense</i> (Fabaceae)	–	Unger and Frank, 2004
<i>Zingiber officinale</i> (Zingiberaceae)	–	Kimura et al., 2010

+, Enzyme induction; –, Enzyme inhibition; NE, No Effect.

CYP2C19

Cytochrome P450 2C19 is not only involved in the metabolism of a range of drugs but it also plays a crucial role in the detoxification and inactivation of some potential carcinogens (Wang et al., 2013). CYP2C19 is responsible for the metabolism of about 10% of prescribed drugs, including a number of essential ones such as the proton pump inhibitor Omeprazole, the tricyclic antidepressant Amitriptyline, the selective serotonin reuptake inhibitor Fluoxetine, the benzodiazepine Diazepam and the barbiturate Phenobarbital (Zhou et al., 2009). About 24 variants of CYP2C19 are known of which three of them were found by REFARGEN in the Brazilian population: CYP2C19*2, CYP2C19*3, and CYP2C19*17. The alleles *2 and *3 appear not to effect the enzyme activity, whereas CYP2C19*17 increases *in vitro* activity and is the most prevalent.

The plant species *Allium sativum*, *Eucalyptus globulus*, *Mentha piperita*, and *Trifolium pratense* were found to be CYP2C19 inhibitors (Table 3). Due to the extensive use of these herbal medicines that are metabolized by CYP2C19, herb-drug interactions at this level could be very frequent but so far no clinical report has been found.

CYP2D6

Although the level of expression of the cytochrome P450 2D6 in the human liver is only about 5%, it metabolizes about 25% of all medications in human liver (Ionescu and Caira, 2005). Essential drugs such as the beta-blockers Propafenone and Timolol, the antidepressant Amitriptyline, the antipsychotic Haloperidol and Risperidone, and the antihistamine Chlorphenamine are metabolized by this isoform (Zhou et al., 2009). Polymorphisms on CYP2D6 are the most studied among the CYPs. To date, 72 variants of this isoform were found in humans and 16 were detected in Brazilians according to REGARGEN: CYP2D6*1, *2, *3, *4, *5, *9, *10, *17, *29, *34, *35, *39, *41, *1XN, *2XN, and *4XN. An increase in enzyme activity was found on the alleles *1XN and

Table 3 | Medicinal plant species listed in RENISUS with reported effects of on CYP2C19.

Plant species/Family	Effects on CYP2C19	References
<i>Allium sativum</i> (Aliaceae)	—	Foster et al., 2001
<i>Eucalyptus globulus</i> (Myrtaceae)	—	Unger and Frank, 2004
<i>Harpagophytum procumbens</i> (Pedaliaceae)	NE	Modarai et al., 2011
<i>Mentha piperita</i> (Lamiaceae)	—	Unger and Frank, 2004
<i>Trifolium pratense</i> (Fabaceae)	—	Unger and Frank, 2004

+, Enzyme induction; —, Enzyme inhibition; NE, No Effect.

*2XN, whereas a decrease of activity was found on *9, *10, *17, *29, and *41.

The popular medicinal plants *Eucalyptus globulus*, *Harpagophytum procumbens*, *Mentha piperita*, *Phyllanthus amarus*, *Punica granatum*, and *Trifolium pratense* are shown to inhibit the activity of CYP2D6 in liver cells (Table 4). Brazilians who take these herbal drugs may have increased chances of ADRs, although no clinical reports were found in the literature.

CYP2E1

The cytochrome P450 2E1 represents 10% of the total CYPs expressed in the human liver and it is well known for its involvement in the metabolism of ethanol to acetaldehyde, and accordingly it is rapidly induced after ethanol ingestion (Anzenbacher and Anzenbacherova, 2001). This cytochrome isoform is responsible for the activation of some carcinogens, procarcinogens and toxicants and it metabolizes mainly low-molecular-weight compounds. CYP2E1 also has the ability to produce reactive intermediates, leading to the formation of free radicals such as superoxide, hydroxyl radical, and lipid peroxides (Neafsey et al., 2009). Polymorphisms on CYP2E1 in the Brazilian population have not yet been mapped by REFARGEN, but one case of herb-drug interaction involving this enzyme has been reported (Hau et al., 2009). According to our literature search, *Allium sativum*, *Momordica charantia*, *Phyllanthus amarus*, *Phyllanthus urinaria*, and *Punica granatum* decrease levels and activity of CYP2E1 in the liver (Table 5). As Acetaminophen is also metabolized by CYP2E1, NAPQI can be also formed at this metabolic route. Therefore, consumption of these medicinal plants could reduce the formation of the toxic metabolite. For example, species of the *Phyllanthus* genus are traditionally used for conditions such as jaundice, gonorrhea, frequent menstruation, diabetes and as a pain killer (Naaz et al., 2007; Patel et al., 2011). A study revealed that *Phyllanthus urinaria* inhibits CYP2E1 activity in hepatocytes and it also attenuates acetaminophen induced hepatotoxicity in mice. The experiment was conducted by treating a total of 37 mice with acetaminophen at a dose of 550 mg/kg of body weight on day one in order to induce liver injury. The mice were then divided into two groups: the first group was treated with *Phyllanthus*

Table 4 | Medicinal plant species listed in RENISUS with reported effects of on CYP2D6.

Plant species/Family	Effects on CYP2D6	References
<i>Allium sativum</i> (Aliaceae)	NE	Markowitz et al., 2003
<i>Eucalyptus globulus</i> (Myrtaceae)	—	Unger and Frank, 2004
<i>Harpagophytum procumbens</i> (Pedaliaceae)	NE, —	Modarai et al., 2011
<i>Mentha piperita</i> (Lamiaceae)	—	Unger and Frank, 2004
<i>Phyllanthus amarus</i> (Euphorbiaceae)	—	Hari Kumar and Kuttan, 2006
<i>Punica granatum</i> (Lythraceae)	—	Usia et al., 2006
<i>Trifolium pratense</i> (Fabaceae)	—	Unger and Frank, 2004

+, Enzyme induction; —, Enzyme inhibition; NE, No Effect.

Table 5 | Medicinal plant species listed in RENISUS with reported effects of on CYP2E1.

Plant species/Family	Effects on CYP2E1	References
<i>Allium sativum</i> (Aliaceae)	—	Le Bon et al., 2003
<i>Curcuma longa</i> (Zingiberaceae)	NE	Salama et al., 2013
<i>Glycine max</i> (Leguminosae)	NE	Shon and Nam, 2004
<i>Momordica charantia</i> (Cucurbitaceae)		Raza et al., 1996
<i>Phyllanthus amarus</i> (Euphorbiaceae)		Hari Kumar and Kuttan, 2006
<i>Phyllanthus urinaria</i> (Euphorbiaceae)		Shen et al., 2008
<i>Punica granatum</i> (Lythraceae)		Faria et al., 2007a

+, Enzyme induction; —, Enzyme inhibition; NE, No Effect.

urinaria extract from day 2 to 4 whereas the second group just received water. The final results indicated that the herbal drug was able to inhibit the formation of NAPQI and, consequently, prevent liver failure (Hau et al., 2009).

CYP3A

The most abundant subfamily of cytochromes is CYP3A (it represents about 30% of the entire CYP450 enzymes in the liver) and it is responsible for processing more than 50% of therapeutic drugs. CYP3A exists in the body in three isoforms: 3A4, 3A5, and 3A7. CYP3A5 is more often detected in adolescents than in adults, where it is hardly inducible. CYP3A4 is mostly glucocorticoid-inducible and CYP3A7 (found only in fetal livers) has a role in hydroxylations of allylic and benzylic carbon

atoms (Ionescu and Caira, 2005). According to our findings, practically all the medicinal plants that demonstrated activity in the Phase 1 metabolism are substrates for the CYP3A family (Table 6). Macrolide antibiotics, anti-arrhythmics, benzodiazepines, immune modulators, HIV antivirals, antihistamines, calcium channel blockers and HMG CoA reductase inhibitors are examples of classes of medications metabolized by the CYP3A subfamily (Zhou et al., 2009). The probability of herb-drug interactions with this isoform is high and therefore a particular attention to all CYP3A substrates should be given in order to avoid herb-drug interactions.

HIV positive patients are commonly treated with the essential drug Saquinavir. At the same time, dietary supplements such as garlic and/or immune system boosters like Cat's claw can be used to help prevent, combat and improve health. In 1998, a case report was published demonstrating that Saquinavir is a substrate of CYP3A4 and that garlic (an inhibitor of CYP3A) was able to interfere with the metabolism of the drug, leading to failures in therapy and possible drug resistance (Chen et al., 2011). Another case report showed that a 45-year-old woman, who was HIV positive and had cirrhosis caused by a hepatitis C infection was not responding satisfactorily to the treatment of the anti-HIV drugs.

Table 6 | Medicinal plant species listed in RENISUS with reported effects of on CYP3A.

Plant species/Family	Effects on CYP3A	References
<i>Allium sativum</i> (Asteraceae)	NE, —(*/*/*/*)	Foster et al., 2001; Hajda et al., 2010
<i>Chamomilla recutita</i> (Asteraceae)	—(*)	Budzinski et al., 2000
<i>Curcuma longa</i> (Zingiberaceae)	NE(*)	Graber-Maier et al., 2010
<i>Eucalyptus globulus</i> (Myrtaceae)	—(*)	Unger and Frank, 2004
<i>Foeniculum vulgare</i> (Apiaceae)	—(*)	Subehan et al., 2006, 2007
<i>Harpagophytum procumbens</i> (Pedaliaceae)	NE, —(*)	Unger and Frank, 2004; Modarai et al., 2011
<i>Mentha piperita</i> (Lamiaceae)	—(*)	Unger and Frank, 2004
<i>Mormodica charantia</i> (Cucurbitaceae)	—(*)	Raza et al., 1996
<i>Phyllanthus amarus</i> (Euphorbiaceae)	—(*/*/*/*)	Hari Kumar and Kuttan, 2006
<i>Punica granatum</i> (Lythraceae)	—(*/*/*/*)	Faria et al., 2007a
<i>Trifolium pratense</i> (Fabaceae)	—(*)	Budzinski et al., 2000
<i>Uncaria tomentosa</i> (Rubiaceae)	—(*)	Budzinski et al., 2000
<i>Zingiber officinale</i> (Zingiberaceae)	(*)	Kimura et al., 2010

*CYP3A4, **CYP3A5, ***CYP3A7+, Enzyme induction; —, Enzyme inhibition; NE, No Effect.

The patient had no good adherence to the HIV treatment so was then asked whether she was making use of any other medicines, including herbal medicines. Surprisingly, the patient was taking *Uncaria tomentosa* preparation for 2 months, probably to enhance the immune system. She was asked to cease taking of the herbal medicine and after 15 days the C_{min} values of the anti-HIV drugs were normalized. As shown in Table 6, *Uncaria tomentosa* has a high inhibitory capability to CYP3A4 causing an increase in the C_{min} values for antiretroviral agents leading to an increased risk of toxicity. The combined therapy with these two agents has been shown to be a potential risk for HIV patients (Lopez Galera et al., 2008).

Another example of a CYP3A substrate is the HMG CoA reductase inhibitor Atorvastatin. This essential medicine is indicated for patients with dyslipidemia, reducing levels of total cholesterol, low-density lipoprotein cholesterol (LDL), triglycerides, very low-density lipoprotein cholesterol (VLDL) and for increasing high-density lipoprotein cholesterol (HDL) levels. A pharmacokinetic study conducted in 2012 showed that the half-life of the drug was increased in rats treated with *Allium sativum* due to inhibition of CYP3A4 by the herbal medicine (Reddy et al., 2012).

REFARGEN has reported CYP3A5 polymorphisms among Brazilians with the most frequent alleles being CYP3A5*1, *3, *6, and *7. The alleles *3 and *6 decrease enzyme activity *in vitro*, whereas *1 and *7 showed no effects (Zhou et al., 2009). Interestingly, the allele *3 is the most common variation of this isoform in Brazil. Because it reduces the enzyme activity, intake of herbal medicines that exert the same effect should be strongly avoided in order to prevent herb-drug interactions.

PHASE 2 METABOLISM AND POTENTIAL PHARMACOKINETIC HERB-DRUG INTERACTIONS

Phase 2 metabolism reactions (or Conjugation reactions) occur when metabolic enzymes react with functional groups of a drug that was formed during the Phase 1 process. Endogenous species, such as a sugar or an amino acid, are added to the drug in order to increase the polarity to allow its elimination. The two main Phase 2 biotransformation reactions are glutathione conjugation and glucuronidation but the other conjugative reactions such as sulfonation, methylation and acetylation are also relevant (Atkinson, 2012).

GLUTATHIONE CONJUGATION (GSH)

Glutathione is a tripeptide present in high concentrations in the liver. It has a protective role removing toxic electrophilic compounds from the body (Ionescu and Caira, 2005). Conjugation with glutathione avoids the reaction of electrophilic compounds to nucleophilic ones in macromolecules such as proteins and nucleic acids. When the conjugate is formed, it has to undergo further metabolic reactions in order to form mercapturic acid. The final product is then eliminated from the organism (Sies and Ketterer, 1988).

The plant species on Table 7 can affect the glutathione levels in liver cells according to our literature search. The metabolite NAPQI, generated by the CYP isoforms 2E1, 3A4, and 1A2, undergoes glutathione conjugation (Baxter and Stockley, 2008).

Table 7 | Medicinal plant species listed in RENISUS with reported effects of on glutathione levels.

Plant species/Family	Effects on glutathione levels	References
<i>Achillea millefolium</i> (Asteraceae)	+	Potrich et al., 2010
<i>Allium sativum</i> (Aliaceae)	+	Ip and Lisk, 1997
<i>Aloe vera/Aloe barbadensis</i> (Aloaceae)	–, +	Kaithwas et al., 2011; Hegazy et al., 2012
<i>Anacardium occidentale</i> (Anacardiaceae)	+	Singh et al., 2004
<i>Baccharis trimera</i> (Asteraceae)	–	Nogueira et al., 2011
<i>Bauhinia forficata</i> (Caesalpinaceae)	–	Damasceno et al., 2004
<i>Bauhinia variegata</i> (Caesalpinaceae)	+	Rajkapoor et al., 2006
<i>Calendula officinalis</i> (Asteraceae)	+	Preethi and Kuttan, 2009
<i>Chamomilla recutita</i> (Asteraceae)	+	Al-Hashem, 2010
<i>Croton cajucara</i> (Euphorbiaceae)	+	Rabelo et al., 2010
<i>Curcuma longa</i> (Zingiberaceae)	+	Rong et al., 2012
<i>Cynara scolymus</i> (Asteraceae)	+, NE	Miccadei et al., 2008
<i>Foeniculum vulgare</i> (Apiaceae)	+	Zhang et al., 2012
<i>Glycine max</i> (Leguminosae)	+	Barbosa et al., 2011
<i>Mentha pulegium</i> (Lamiaceae)	+	Alpsay et al., 2011
<i>Mentha piperita</i> (Lamiaceae)	+	Sharma et al., 2007
<i>Mikania glomerata</i> (Asteraceae)	NE	Barbosa et al., 2012
<i>Momordica charantia</i> (Cucurbitaceae)	+	Raza et al., 2000, 1996
<i>Phyllanthus amarus</i> (Euphorbiaceae)	+	Kumar and Kuttan, 2004, 2005; Karuna et al., 2009; Maity et al., 2013
<i>Phyllanthus niruri</i> (Euphorbiaceae)	+	Bhattacharjee and Sil, 2006; Manjrekar et al., 2008
<i>Psidium guajava</i> (Myrtaceae)	+	Tandon et al., 2012
<i>Punica granatum</i> (Myrtaceae)	+, –	Faria et al., 2007b; Dassprakash et al., 2012
<i>Ruta graveolens</i> (Rutaceae)	+	Ratheesh et al., 2011
<i>Zingiber officinale</i> (Zingiberaceae)	+, NE	Ajith et al., 2007

+, Enzyme induction; – Enzyme inhibition; NE, No Effect.

When production of NAPQI exceeds liver stores of glutathione, the organ is damaged due to the attachment of NAPQI to liver proteins (Alipour et al., 2013). Thus, a combined therapy of acetaminophen and herbal species that deplete glutathione levels, listed in Table 7, should be monitored.

A study demonstrated that consumption of garlic extract protects hepatocytes against acetaminophen-induced glutathione depletion. In order to measure glutathione levels, hepatocytes were isolated from male Sprague-Dawley rats and incubated with different concentrations of garlic extract at three different times: before, at the same time and 30 min after addition of acetaminophen. The results were collected at different time points (from 0 to 150 min) and it was observed that the intake of garlic extract is able to protect hepatocytes against acetaminophen-induced toxicity by increasing intracellular GSH levels (Anoush et al., 2009).

Studies on polymorphisms in the Brazilian population of enzymes in glutathione conjugation, such as glutathione S-transferase (GST) have not been carried out by REFARGEN. However, some reports revealed its existence among this population. A study conducted by Rossini et al. (2002) reported the existence of GST polymorphisms in a group of 519 Brazilians from Rio de Janeiro. GSTM1 is involved in the detoxification of polycyclic aromatic hydrocarbons and some mutagens, whereas GSTT1 catalyzes the metabolism of halomethanes by human erythrocytes. It was found that the null allele, i.e., no expression of the enzyme, was detected in approximately 10% of the studied population. Null individuals are generally more susceptible to DNA damage by the action of the compounds already mentioned. Although we cannot extrapolate this data for all Brazilians, the existence of these polymorphisms is thought to be quite common among multi-ethnic populations. Therefore, the extensive list of plant species found to affect the glutathione conjugation mechanism plus the presence of such polymorphisms could potentially increase chances of herb-drug interactions.

GLUCURONIDATION

Glucuronidation is a mechanism in which a glucuronide is formed by the reaction between the electrophilic C-1 atom of the pyranose acid ring of the co-factor UDPGA (uridine 5'-diphosphate-glucuronic acid) with the substrate catalyzed by UDP-glucuronosyltransferases (UGTs). Uridine diphosphate glucuronosyltransferases (UGT's) are the most important Phase 2 enzymes and they are found in the highest amount among all conjugation enzymes in the liver (Ionescu and Caira, 2005; Caira and Ionescu, 2006). This is the most important form of conjugation of xenobiotics with chemical groups such as alcohols, phenols, hydroxylamines, carboxylic acids, amines, sulphonamides, and thiols (Gibson and Skett, 2001).

Phase 1 metabolites of nonsteroidal anti-inflammatory drugs (NSAIDs), such as Ibuprofen, predominantly undergo glucuronidation in order to be eliminated from the organism (Kuehl et al., 2005). *Allium sativum* increases the expression of UGTs whereas *Curcuma longa* inhibits the expression of these enzymes in the liver (Table 8). Therefore, the pharmacokinetic of this class of drugs can be compromised by consumption of any of these herbal species.

REFARGEN has not yet published data about polymorphisms on UDP-glucuronosyltransferase enzymes. However, information about polymorphic UGTs and its impact on ADRs and cancer susceptibility have been reported (Guillemette, 2003).

OTHER CONJUGATIVE REACTIONS

Besides the two main liver conjugation reactions (glutathione conjugation and glucuronidation), the xenobiotic transformation could also happen by sulfation (or sulfate conjugation), methylation (or methyl conjugation), and acetylation (Ionescu and Caira, 2005).

Sulfation is another Phase 2 detoxification mechanism that is recognized to be the major conjugation pathway for phenols, alcohols, amines and thiols (Ionescu and Caira, 2005). Before the Phase 1 metabolites undergo sulfate conjugation, the inorganic sulfate has to be activated via ATP to form adenosine-5'-phosphosulphate (APS) and, consequently, 3'-phosphoadenosine-5'-phosphosulphate (PAPS). Sulfotransferase enzymes (SULT) will then catalyze the detoxification of essential drugs, such as Salbutamol and Acetaminophen by transferring a sulfonyl group from PAPS to an acceptor molecule (Gibson and Skett, 2001). Methylation is the mainly metabolic pathway for endogenous compounds but it could be also the route of many drugs and xenobiotics in general (Weinshilboum, 1988). Methyl conjugate reactions are only possible in the presence of the co-factor S-adenosylmethionine (SAM) and will result in the formation of O-methylated, N-methylated, and S-methylated products (Gibson and Skett, 2001). The liver is the primary site for acetylation reactions, but the reaction could also happen in some extra hepatic sites, such as the spleen, lungs and gut. To summarize, the N-acetyltransferases will catalyze the transferring of the co-factor acetyl-coenzyme A (acetyl-CoA) to aromatic amines and sulphonamides and form the polar metabolites (Ionescu and Caira, 2005).

After the literature search, it was concluded that the 71 plant species that are the focus of this review need to have their pharmacokinetic profile studied, because no such data has been published. The risk of potential herb-drug interactions caused by herbal medicines that are metabolized through sulfation, methylation and acetylation pathways is therefore increased and it could be already affecting the efficacy of conventional drugs. REFARGEN has not published any data about genetic polymorphisms in the Brazilian population for these conjugation mechanisms to date.

Table 8 | Medicinal plant species listed in RENISUS with reported effects of on UGT levels.

Plant species/Family	Effects on UGT levels	References
<i>Allium sativum</i> (Aliaceae)	+	Ip and Lisk, 1997
<i>Curcuma longa</i> (Zingiberaceae)	—	Naganuma et al., 2006

+, Enzyme induction; —, Enzyme inhibition; NE, No Effect.

EFFECTS OF PLANT SPECIES ON P-GLYCOPROTEIN (Pgp) ACTIVITY AND HERB-DRUG INTERACTION

Drug-transporter proteins are known to allow xenobiotics to cross biological membranes, the most well-known one being Pgp. This protein plays a role as an efflux pump that pushes metabolites and drugs out of the cells which can result in pharmacokinetic alterations (Williamson et al., 2009). Some metabolites generated by the metabolism of herbal medicines can be pumped back to the lumen due to the activity of P-glycoprotein and hence, oral delivery can be compromised (Butterweck et al., 2004). Among the herbal medicines that are the object of this review, *Achillea millefolium* demonstrated inhibition of P-glycoprotein whereas *Allium sativum* activates the transporter (Table 9). Herb-drug interactions have already been reported on Pgp activity. For example, the HIV-protease inhibitor Saquinavir (substrate of CYP3A4) is absorbed in the intestine via P-glycoprotein. As garlic extracts may induce Pgp activity, the outcome of concomitant consumption of the herbal medicine with the essential drug could potentially reduce the bioavailability of the latter (Williamson et al., 2009).

Studies have shown that the gene ABCB1, which encodes Pgp, is very polymorphic and that the pharmacokinetics of several Pgp substrates could be significantly altered (Scheiner et al., 2010). REFARGEN has identified three ABCB1 polymorphisms in the Brazilian population: 1236T (rs1128503), 2677non G (rs2032582), and 3435T (rs1045642). Pharmacokinetic studies of drugs that affect the regulation of ABCB1, will help in the future in order to avoid herb-drug interactions on that level.

PHARMACOVIGILANCE OF HERBAL MEDICINES

The increasing consumption of herbal medicines in the world raises a concern about their rational use by the population. The WHO has recognized the potential risks of uncontrolled use of herbal medicines in conjunction with other medicines and hence, this Organization issued in 2004 the "Guidelines on safety monitoring of herbal medicines in pharmacovigilance systems." These guidelines indicate how member countries should include herbal medicines to an existent pharmacovigilance system to facilitate the exchange of information (WHO, 2004). After the establishment of ANVISA in 1999, efforts were concentrated on the creation of a national system of pharmacovigilance, and in 2001 the National Center for Drug Monitoring (CNMM) was founded. Notifications of ADRs in Brazil is currently made through an

Table 9 | Medicinal plant species listed in RENISUS with reported effects of on P-glycoprotein activity.

Plant species/Family	Effects on P-glycoprotein activity	References
<i>Achillea millefolium</i> (Asteraceae)	—	Haidara et al., 2006
<i>Allium sativum</i> (Aliaceae)	+	Hajda et al., 2010
<i>Curcuma longa</i> (Zingiberaceae)	NE	Graber-Maier et al., 2010

+, Efflux increased; —, Efflux decreased.

electronic system called NOTIVISA and these data come from three different sources: a “Sentinel Network,” which provides information on adverse reactions related to the use of health products in hospitals, such as drugs, blood and hemoderivatives; pharmacies will report suspected cases of drug reactions (including herb-drug interactions); and a scheme of “Spontaneous Notifications” that can be made by any health professional registered into the electronic system (Mendes et al., 2008).

The number of ADRs notified by pharmacovigilance systems in the world until 2012 resulting from herb-drug interactions was 811, with *Allium sativum*, *Mentha piperita*, *Zingiber officinale* and *Glycine max* among the top 20 of the most commonly reported herbal medicines. Brazil was not included in the list of countries which have reported herb-drug interactions, demonstrating the difficulties in collection of such data by the pharmacovigilance system (Skalli and Soulaymani Bencheikh, 2012). Since the creation of NOTIVISA in 2008, there are no notifications of adverse reactions to herbal medicines, demonstrating that the new system created more obstacles to the users (Balbino and Dias, 2010).

Most of the problems related to the notification of ADRs due to herbal medicine consumption occur mainly because Practitioners and other health professionals are not well trained to detect the origin of the reaction. Besides, patients in general do not inform their use of herbal medicines and Physicians may not have sufficient knowledge about the effects of the phytomedicines in the body. Thus, educational campaigns should be utilized in order to emphasize the rational use of medicinal plants and also to encourage health professionals to notify any adverse effect that might be a result of herb-drug interaction (Balbino and Dias, 2010).

CONCLUSIONS

In 2008 Brazil became a model country, clearly defining a positive list of herbal medicines considered “essential,” and implementing an agenda to make possible the complete implementation of herbal medicines at a clinical level in the foreseeable future. This is a leading example within the current global trend toward the integration of herbal medicines into the healthcare system.

Our review highlights that little is known about native Brazilian plants. Most of the data is related to herbal medicines used worldwide, such as garlic, mint and devil’s claw. We tried to interpret this in the context of the genetic makeup of the Brazilian population and how this may interact with essential drugs prescribed within the Brazilian healthcare system. It is evident that an enormous task should be undertaken to understand the pharmacokinetics of most local plant species.

Ensuring safety of herbal medicines goes beyond the interpretation of the preclinical or clinical evidence. Quality is also on the basis of safety and this is a challenging task in the field of Phytotherapy, with many drugs lacking identifiable active principles. Healthcare professionals and the public alike have to be well trained/informed in the use of the final medicinal product. Moreover, only a robust system of pharmacovigilance will help in the identification of relevant safety issues at a population level. These are not necessarily mirroring the preclinical and clinical data available.

At this stage, the preclinical pharmacokinetic profile of medicinal plants can only be evaluated with available data in literature.

Polymorphism studies in the Brazilian population have been a valuable source of information that can help with the assessment of ADRs and herb-drug interactions.

By tentatively exploring any potential interaction between herbal medicines and other essential medicines in the Brazilian system, we hope to open up future research to both bridge the gaps in knowledge and support future risk assessment of herb-drug combinations. Again, our results highlight that much more work is needed as over half of the medicinal plants lack data. Only after this is done can we start assessing the risk of herb-drug interactions when medicinal plants are taken with any essential drug.

Safety of herbal medicines finally lies in the hands of the patients. In this regard, healthcare professionals need proper training on how to advise the patient and the labeling of the product must anticipate further doubts when the patient cannot communicate with the professional.

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7.10.3 *In vitro* effects of four native Brazilian medicinal plants in CYP3A4 mRNA gene expression, glutathione levels, and P-glycoprotein activity



In vitro Effects of Four Native Brazilian Medicinal Plants in CYP3A4 mRNA Gene Expression, Glutathione Levels, and P-Glycoprotein Activity

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Erythrina mulungu Benth. (Fabaceae), *Cordia verbenacea* A. DC. (Boraginaceae), *Solanum paniculatum* L. (Solanaceae) and *Lippia sidoides* Cham. (Verbenaceae) are medicinal plant species native to Brazil shortlisted by the Brazilian National Health System for future clinical use. However, nothing is known about their effects in metabolic and transporter proteins, which could potentially lead to herb-drug interactions (HDI). In this work, we assess non-toxic concentrations (100 µg/mL) of the plant infusions for their *in vitro* ability to modulate CYP3A4 mRNA gene expression and intracellular glutathione levels in HepG2 cells, as well as P-glycoprotein (P-gp) activity in vincristine-resistant Caco-2 cells (Caco-2 VCR). Their mechanisms of action were further studied by measuring the activation of human pregnane X receptor (hPXR) in transiently co-transfected HeLa cells and the inhibition of γ-glutamyl transferase (GGT) in HepG2 cells. Our results show that P-gp activity was not affected in any case and that only *Solanum paniculatum* was able to significantly change CYP3A4 mRNA gene expression (twofold decrease, $p < 0.05$), this being correlated with an antagonist effect upon hPXR ($EC_{50} = 0.38$ mg/mL). Total intracellular glutathione levels were significantly depleted by exposure to *Solanum paniculatum* (−44%, $p < 0.001$), *Lippia sidoides* (−12%, $p < 0.05$) and *Cordia verbenacea* (−47%, $p < 0.001$). The latter plant extract was able to decrease GGT activity (−48%, $p < 0.01$). In conclusion, this preclinical study shows that the administration of some of these herbal medicines may be able to cause disturbances to metabolic mechanisms *in vitro*. Although *Erythrina mulungu* appears safe in our tests, active pharmacovigilance is recommended for the other three species, especially in the case of *Solanum paniculatum*.

Keywords: herbal medicines, Brazil, glutathione, CYP3A4, P-Glycoprotein, drug metabolism

INTRODUCTION

According to the World Health Organization (WHO), 65–80% of the world's population in developing countries depends on medicinal plants for their primary health care due to poverty or lack of access to modern medicine (Silveira et al., 2008). Latin American countries possess an enormous part of the world's biodiversity, with Brazil alone containing approximately 22% of all

existing plants and microorganisms on earth (Calixto, 2005). For the past 10 years, Brazilian health care authorities have directed their attention to the considerable use of medicinal plants in hope to integrate traditional Brazilian medicine into the public health care system (SUS). Indeed, there is a growing demand for the use of medicinal plants by the citizens of Brazil as well as an interest in natural products to support healthier lifestyles (Silveira et al., 2008).

Since 2004, the SUS, along with the Ministry of Health (MoH), have created a National Policy on Integrative and Complementary Practices (PNPIC), which is aimed at offering traditional medicinal plants as a treatment option through the SUS with guaranteed safety and quality (Balbino and Dias, 2010). The MoH and the SUS surveyed municipalities nationwide and, using indications according to categories of the International Classification of Diseases, created the National List of Medicinal Plants of Interest to SUS (RENISUS)¹. This list is composed of 71 plants, which have the potential to generate products of interest for the SUS, as well as promote the traditional practice of herbal remedies. In the same year, the Brazilian Health Surveillance Agency (ANVISA) began to regulate the registration of herbal medicines, requiring quality control and safety reports confirming good manufacturing practice for all herbal medicines (Oliveira et al., 2006). As a result of this advancement, the first phytomedicine to be fully developed in Brazil was approved by ANVISA. It is an anti-inflammatory topical ointment marketed as Acheflan®, in which the active ingredient is the Brazilian medicinal plant *Cordia verbenacea* DC (Boraginaceae), a plant analyzed in this study (Calixto, 2005).

Plant species included in the RENISUS list are prioritized to undergo safety and efficacy studies. To date, very little is known about the effects they may cause on phase 1 and phase 2 metabolism and transporter proteins. Also, the pharmacokinetic (PK) profile of herbal medicines is virtually impossible to study as they are Complex Chemical Entities, i.e., consisting of numerous chemicals with disparate absorption rates, pharmacodynamic (PD) and PK properties (He et al., 2011). Therefore, the preclinical approach should clarify whether herbal medicines can alter the activity and expression of discrete metabolic and/or transporter proteins in order to theorize which prescription drugs (single chemical entities) would be affected by their concomitant use.

Since CYP3A4 metabolizes most of the currently marketed drugs, glutathione is a central player in drug conjugation and *P*-glycoprotein (*P*-gp) activity determines drug absorption, most of the preclinical safety studies of natural and synthetic drugs focus on these targets (Zhou, 2008; Glaeser, 2011; Lu, 2013).

Such data is necessary in order to predict and avoid interactions that may occur between the extracts and conventional drugs. These types of interactions are called herb-drug interactions (HDI) (Mazzari and Prieto, 2014b). In a previous literature review, we revealed that such data are known for only half of the medicinal plants traditionally used in Brazil

(Mazzari and Prieto, 2014a). In order to fill this information gap, we embarked on a study of 24 medicinal plants for which no information of this sort was found. Here we report on our results for four native plants on RENISUS, namely *Erythrina mulungu* Benth. (Fabaceae), *Cordia verbenacea* A. DC. (Boraginaceae), *Solanum paniculatum* L. (Solanaceae) and *Lippia sidoides* Cham. (Verbenaceae), which are used in the treatment of symptoms ranging from anxiety to gastric dysfunctions (Table 1).

MATERIALS AND METHODS

Chemicals and Reagents

Cell Culture

HepG2 and Caco-2 cell lines were from Sigma Aldrich at passages 100 and 43 respectively. HeLa cells were donated by Dr. Paul Webb from the Houston Methodist Institute for Technology at passage 20. All cell culture reagents were from Gibco® Invitrogen unless otherwise stated. Vincristine 2 mg/mL was purchased from Hospira Ltd.

HPTLC Analysis

Water, Dichloromethane (>99.8%, contains amylene as a stabilizer), ethyl acetate (>99.7%) and methanol (>99.9%) all ChromasolvPlus for HPLC provided by Sigma Aldrich. Acetic acid (glacial) analytical reagent grade provided by Fisher Scientific. Formic Acid 98% provided by Rectapur® VWR.

Caffeic acid was from Kotch-light Laboratories LTD. Rutin hydrate, quercetin dehydrate and gallic acid all from Sigma Aldrich. Diphenylborinic acid 2-aminoethyl ester (98%) and coumarin laser grade (99% UV-Vis) were provided by ACROS organics. Luteolin (HPLC grade) was from Extrasynthèse. Chlorogenic acid provided by Cayman Chemical Co. Polyethylene glycol 4000 grade was from Fisher Scientific.

Cell Viability Assays

Sulphorhodamine B (SRB), Neutral Red (NR), (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT), trichloroacetic acid (TCA), glacial acetic acid, 96% ethanol, hydrochloric acid (HCl), Isopropanol and Tris base were from Sigma Aldrich.

CYP3A4 mRNA Gene Expression Assay

Oligonucleotide primers were custom-synthesized by Invitrogen Life Technologies and Sigma Aldrich. TRIzol® (Total RNA Isolation Reagent), Oligo (dT) 12-18 primers, M-MLV Reverse Transcriptase, RNaseOUT, DNase I Amplification Grade and 100 mM dNTP Set were purchased from Invitrogen Life Technologies. SYBR Green (2xqPCR Master Mix premixed with SYBRgreen) was obtained from Amethyst reagents (Cambridge Bioscience). Rifampicin and DMSO were purchased from Sigma Aldrich.

hPXR Assay

Lipofectamine® 2000 was purchased from Invitrogen. Rifampicin and Luciferase assay system were obtained from Sigma Aldrich and Promega, respectively.

¹SUS (2009). *Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS)*, Brasília. Available at: http://bvsms.saude.gov.br/bvs/sus/pdf/marco/ms_relacao_plantas_medicinais_sus_0603.pdf [accessed February 22, 2016].

TABLE 1 | Botanical, pharmacopoeial, pharmacological and phytochemical information of the four Brazilian native species.

Family	Scientific name	Popular name	Brazilian pharmacopoeia edition	Part	Traditional indication and experimental use	Pharmaceutical formulation	Chemistry
Leguminosae	<i>Erythrina mulungu</i> Mart. Ex Benth	Mulungu/Mulungu/ Suina/Sapalinho de judeu/Bico de papagaio (Brandão et al., 2006)	1st (1926)/2nd (1959) (Brandão et al., 2006)	Wood (Brandão et al., 2006)	Mild sedative/Insomnia/ Depression (Setti-Perdigão et al., 2013)	Fluid-extract/Tincture/Vegetal drug (Brandão et al., 2006)	Alkaloids (+)-1 ta-hydroxy-erythrine/Erythrine/(+)- α -hydroxyerythrine (Flausino et al., 2007)
Solanaceae	<i>Solanum paniculatum</i> L.	Jurubeba/Juripeba/Jubeba/Jubena/Juuna (Brandão et al., 2006)	1st (1926) (Brandão et al., 2006)	Roots (Brandão et al., 2006)	Tonic/Anti-fever agent/Coldague/Bitter/Euaptic to treat liver and gastric dysfunctions (Mesa-Vela et al., 2002; Martins et al., 2015; Vieira Junior et al., 2015)	Tincture/Dry Extract/Fluid-Extract/Vegetal Drug (Brandão et al., 2006)	Flavonoids (Matias et al., 2016) Alkaloids (Jurubebina, Jubebine, Solanine) Resins (Jupabina, Jupeberina) Saponins (Isolupidine, Isolubidine, Isopaniculidine and Jurubidine) (Mesa-Vela et al., 2002)
Verbenaceae	<i>Lippia sidoides</i> Cham.	Alecrim-pimenta (ANVISA, 2011)*	Formulário de Fitoterápicos da Farmacopéia Brasileira – 1ª Edição – 2011 (ANVISA, 2011)*	Leaves (ANVISA, 2011)*	Herpes Virus (HHV-1) (Vladares et al., 2009) Chemoprevention agent (Ernst et al., 2010) Anti-septic/Anti-microbial (Almeida et al., 2010)	Extemporaneous preparations/Tincture/Gel/Soap (ANVISA, 2011)*	Flavonoids/Quinones/Triterpenes/Lignanes/Free and glycosylated steroids/Organic acids (Almeida et al., 2010)
Boraginaceae	<i>Cordia verbenaceae</i> DC.	Erva – baleira (ANVISA, 2011)*	Formulário de Fitoterápicos da Farmacopéia Brasileira – 1ª Edição – 2011 (ANVISA, 2011)*	Leaves (Ventrella and Marinho, 2008; ANVISA, 2011)*	Anti-inflammatory/Analgesic/Anti-ulcerogenic/healing agent (Ventrella and Marinho, 2008)	Tea/Infusions (Ventrella and Marinho, 2008)	Monoterpenes/Sesquiterpenes/Triterpenes/Flavones (Ventrella and Marinho, 2008)

*ANVISA, A.N.D.V.S. (2011). Formulário de Fitoterápicos – Farmacopéia Brasileira, Brasília. Available at: http://www.anvisa.gov.br/hotsite/farmacopelabrasileira/conteudo/Formulario_de_Fitoterpicos_da_Farmacopela_Brasileira.pdf [accessed June 16, 2013].

Intracellular Glutathione Assay

Buthionine sulfoximine (BSO), L-glutathione reduced, glutathione reductase, 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB), β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH), Triton-X and sulfosalicylic acid were from Sigma Aldrich.

GGT Activity Assay

L-Glutamic acid γ -(p-nitroanilide) hydrochloride, 4-nitroaniline, glycyl-glycine (Gly-Gly), Tris base and acivicin were from Sigma Aldrich.

Rhodamine 123 Uptake Assay

Rhodamine 123 was from Sigma Aldrich. Verapamil (Securon IV 2.5 mg/mL) was from Abbott Laboratories Ltd.

Plant Materials and Extraction

Plant materials were collected from "Farmácia Viva Brasília" (Brasília, DF – Brazil); via the University of Brasília – UnB. The plants were grown and processed according to good practices, ensuring validity and quality².

All whole plant handling and extraction were performed at UnB. Aerial parts of the plants (100 g) were subjected to a 20 min infusion, to mimic traditional use. The infusions were filtered, lyophilized, and immediately sent to UCL School of Pharmacy. The extracts were stored at -18°C throughout the studies.

Special permission must be granted from the Brazilian Council for Management of Genetic Heritage (CGEN) in order to have access to genetic material, respecting international intellectual and genetic property rights laws. Access was authorized (license n. 010295/2014-3) resulting from collaboration between University College London (UCL) and UnB. Access was granted on May 2014, and the plant extracts were received on June 2014.

HPTLC Analysis

Extracts were diluted to a concentration of 50 mg/mL in methanol. Control compounds were made at a concentration of 1mg/mL, also diluted in methanol. A CAMAG Linomat 5 was used to apply 5 μL of the samples to TLC silica gel 60 F254 aluminum sheets. The plates were developed using a CAMAG ADC2 automatic developing chamber. The method included 30-s pre-drying, 10min humidity control using magnesium chloride to 48.3% relative humidity and 20 min saturation time, using saturation pads all done at 25.2°C . The mobile phase used was ethyl acetate: formic acid: water (82:9:9). During development, the solvent front was allowed to migrate 80 mm before a drying time of 5 min. For derivatization, we used Natural products reagent (NPR) followed by PEG 4000 (Reich and Schibli, 2007). All visualization and analysis were done using CAMAG TLC visualizer both before and after derivatization.

²SUS (2010). *Ministério da Saúde. Portaria 886, de 20 de abril de 2010. Institui a Farmácia Viva no âmbito do Sistema Único de Saúde*. Available at: http://bvsms.saude.gov.br/bvs/saudelegis/gm/2010/prt0886_20_04_2010.html [accessed February 22, 2016].

Cells Culture

HepG2 cells were cultured in Minimal Essential Medium (MEM) Alpha supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin. Caco-2 cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) with high glucose (4.5g/L) and L-glutamine supplemented with 10%FBS, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, 1% non-essential amino acids (NEEA) and Vincristine (50 μM). HeLa cells were cultured in DMEM media supplemented with 10% FBS, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin. All cell lines were kept in the NuAire DH Autoflow CO_2 Air-Jacketed incubator at $37^{\circ}\text{C}/5\%\text{CO}_2$.

Cell Viability Assays

The SRB, NR and MTT assays were performed as previously described (José Ruiz et al., 2006; Houghton et al., 2007; Repetto et al., 2008).

Real-Time RT-qPCR Analysis

mRNA Extraction and cDNA Synthesis

After exposing HepG2 cells (5×10^5 cells/well) to plant extracts or the CYP3A4 inducer Rifampicin (50 μM) or the CYP 3A4 inhibitor DMSO 1% for 96 h, total RNA was extracted from using TRIzol[®] Reagent according to the manufacturer's protocol. Samples were treated with DNase I (1 U/ μL) to avoid genomic contamination. The quantity and quality of RNA was determined by differential readings at 260 and 280 nm in a Nanodrop 2000 (Thermo Scientific). The integrity of total RNA from HepG2 cells was assessed by visual inspection of the two rRNAs 28 and 18 s on agarose gels. cDNA was synthesized from 1 μg of total RNA with the Moloney rine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 U/ μL) and oligo(dT) 12-18 primer (0.5 $\mu\text{g}/\mu\text{L}$), according to the manufacturer's instruction in a final volume of 21 μL .

RT-qPCR Conditions and Analysis

CYP3A4 sense strand primer sequence was 5'-CAAGGACAA CATAGATCGTTACATATACACACCCTTTGGAAG-3' and the antisense strand primer was 5'-AGCTCAATGCATGTACA GAATCCCCGGTTA-3' (Usui et al., 2003). The β -actin gene was used to control for variations in RNA loading within the experimental conditions. The sense strand primer sequence was 5'-CGTACCACTGGCATCGTGAT-3' and the antisense strand primer was 5'-GTGTTGGCGTACAGGTCTTTG-3'. The RT-qPCR was carried out in 96-well plates using a PikorealTM Real-Time PCR detection system (Thermo Scientific). Each well contained a final reaction volume of 10 μL (5.0 μL MasterMix with SYBR Green, 2.0 μL cDNA template diluted appropriately, 0.5 μL of each primer at a final concentration 0.3 mM and 2.0 μL of RNase/DNase free distilled water). PCR reaction was performed under the following schema: initial denaturation at 95°C for 2 min, then 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C (β -actin) or 60°C (CYP3A4) for 30 s, and extension at 72°C for 30 s.

At the end of the run, a melting curve was generated by heating the amplicon from 60 to 95°C in order to confirm the specificity of the amplification for each primer pair. All RT-qPCR were run

in quadruplicates. Standard curves were produced to check the PCR efficiency using a fivefold dilution series of cDNA. Efficiency (E) of primer pairs was obtained from the slope of the calibration curve generated. The relative expression was calculated on the basis of 'delta delta Ct' ($\Delta\Delta C_t$) values. Normalization of the target gene was achieved by using β -actin as a reference gene.

hPXR Activation Assay

After 24 h seeding, HeLa cells (4×10^4 cells/well) were transiently co-transfected with 60 ng of pM-Gal4-PXR-LBD and 240 ng of Gal4 luciferase reporter using lipofectamine 2000 reagent according to the manufacturer's protocol. Transfected cells were treated with increasing concentrations of plant extract and/or rifampicin 1 μ M (EC50). Luciferase activity was measured after 24 h, according to manufacturer's protocol in a 20/20 n Glomax luminometer and reported as a response (%) compared to cells treated only with rifampicin.

Intracellular Glutathione Levels

The method used in the intracellular determination of glutathione levels was adapted from those described by Allen et al. (2000) and Rahman et al. (2006) with slight modifications. After 24 h incubation with BSO (10 μ M) or plant extracts (100 μ g/mL), HepG2 cells (4×10^4 cells/well) were washed with PBS and 60 μ L of 0.1% Triton-X was added to each well of the plates to lyse the cells. Twenty five micro liter of 5% sulfosalicylic acid was added to the cell lysates and plates were shaken for 2 min. Twenty five micro liter of glutathione reaction buffer containing NADPH (2.39 mM), DTNB (0.01 M) and glutathione reductase (500UI) in sodium phosphate buffer (143 mM) containing EDTA (6.3 mM) was added to the cell lysates. Absorbance was read in a kinetic cycle in the plate reader every 30 s for 5 min at 405 nm (11 readings). Absorbances were converted into absolute amounts by means of the 1-slopes method using known concentrations of L-glutathione reduced.

GGT Activity Assay

GGT activity assay was conducted according to Rebbear et al. (1998) with slight modifications. Briefly, after 24 h incubation of HepG2 cells (1×10^6 cells/well) with the GGT inhibitor acivicin (5 μ M) or plant extracts (100 μ g/mL), media was aspirated and cells were washed with PBS. Four milliliter of pre-warmed glycylglycine buffer (115 mM Tris, 138mM glycylglycine) and 400 μ L of the substrate γ -glutamyl-p-nitroanilide (29.6 mg/mL of HCl 0.5 mmol/L) were added to the wells and plates were incubated for 10 min. Then, 500 μ L of the content of each well were transferred to 24-well plates and absorbance was measured in the plate reader (405 nm). Absorbances were converted into absolute amounts by means of a calibration line using 4-nitroaniline.

Rhodamine 123 Uptake Assay

Rhodamine uptake/efflux assays were conducted as described by Chieli et al. (1993) with minor modifications. After five passages in media containing vincristine (50 μ M), Caco-2 VCR (Eneroth et al., 2001) cells (1×10^4 cells/well) were incubated for 2 h

with the P-gp inhibitor verapamil (20 μ M) or plant extracts (100 μ g/mL) in serum-free media containing rhodamine 123 (5 μ g/mL). After incubation, cells were washed with verapamil (20 μ M) in PBS. Cells were lysed with 100 μ L of 0.1% Triton X-100 in PBS and the plates were placed in the incubator for 15 min. The fluorescence intensity of cell lysates was measured in the plate reader (Exc-485 nm, Em-525 nm). The cellular accumulation of rhodamine 123 for each of the extracts was expressed as the percentage of the accumulation measured for rhodamine 123 under control conditions.

Statistical Analysis

Collected data were analyzed as means \pm SD of at least three independent experiments. Statistical significance was measured by student *t*-test and ANOVA followed by Bonferroni correction using GraphPad InStat (GraphPad Software Inc., La Jolla, CA, USA). Results with a value of $p < 0.05$ were considered significant.

RESULTS

Yield of Plant Extracts

Yields of herbal extracts were as follows: *E. mulungu* (5.77%), *Solanum paniculatum* (9.27%), *Lippia sidoides* (13.33%) and *Cordia verbenaceae* (14.95%).

HPTLC Analysis

Cordia verbenaceae fingerprint contains caffeic acid at retention factor (Rf) = 0.87 and chlorogenic acid at Rf = 0.38. *Solanum paniculatum* contains gallic acid at Rf = 0.81, rutin at Rf = 0.20 and chlorogenic acid at Rf = 0.38. *Lippia sidoides* contains luteolin at Rf = 0.86 and minor amounts of quercetin at Rf = 0.90. *E. mulungu* did not contain any of these metabolites in significant amounts (see Figure in Supplementary Material).

Cell Viability

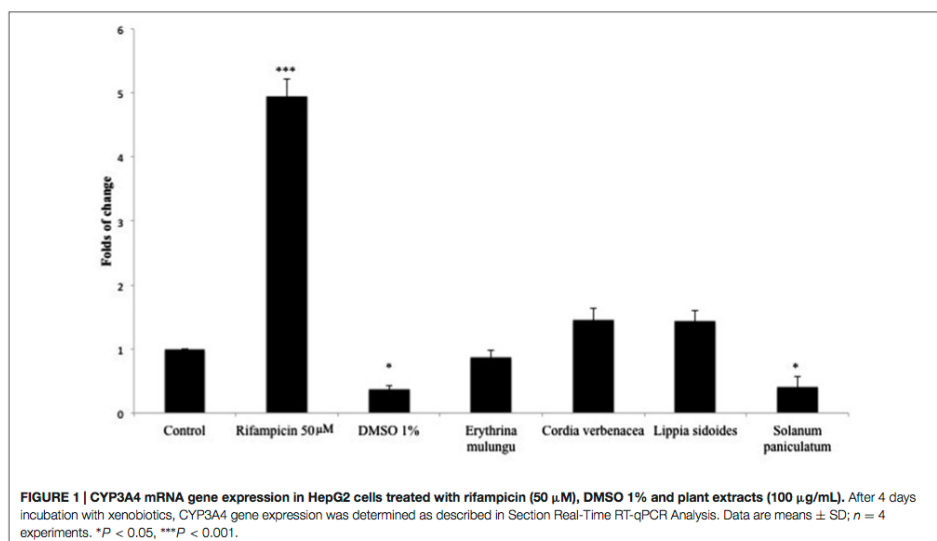
HepG2, Caco-2VCR, and HeLa cells exhibited more than 80% viability after 24 h incubation of 100 μ g/mL of plant extracts. This allowed us to work on all experiments at that non-toxic concentration (Data presented as Supplementary Materials).

Real-Time qPCR Efficiency

Both CYP3A4 and β -actin primers sequences revealed the specificity of target amplification. Baseline and threshold were properly set. Standard curve demonstrated good regression coefficient and efficiency (Data presented as Supplementary Materials). Melting curve analysis revealed a single peak for each pair of primers (Data presented as Supplementary Materials).

CYP3A4 mRNA Gene Expression

Rifampicin (50 μ M), a known CYP3A4 inducer, was able to significantly increase CYP3A4 expression in 4.95 folds ($p < 0.001$). DMSO 1% inhibited CYP3A4 expression in 2.7 folds ($p < 0.01$). *E. mulungu*, *Cordia verbenaceae* and *Lippia sidoides* were not able to modulate CYP3A4 expression in a



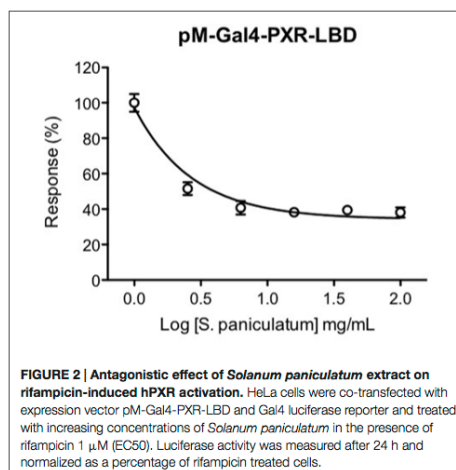
significant manner compared to non-treated cells. However, *Solanum paniculatum* inhibited CYP3A4 mRNA gene expression in 2.4 folds, showing a similar effect to DMSO 1% (*p* < 0.01) (Figure 1).

hPXR Antagonistic Effect of *Solanum paniculatum*

To further investigate whether the diminished expression of CYP3A4 mRNA gene by *Solanum paniculatum* treatment was mediated by an antagonist effect upon hPXR, we performed a reporter gene assay. As we can observe, co-transfected HeLa cells treated with rifampicin (1 µM) and serial dilutions of *Solanum paniculatum* extract showed a dose-response inhibition upon hPXR transcription activity. The maximal inhibition was close to 60% with a IC₅₀ of 0.38 mg/mL (Figure 2).

Inhibition of hPXR reporter gene assay was not due to interference of the extract with the luciferase activity, since it did not show any effect on luciferase activity in HeLa cells transfected with CMV-luciferase expression vector. Additionally, this extract showed an agonist effect in HeLa cells co-transfected with thyroid hormone receptor beta 1 ligand binding domain and Gal4 luciferase reporter treated with triiodothyronine (T3). We did not use *Renilla* luciferase assay as an internal control since several authors have been describing the limitations of *Renilla* luciferase as an internal control of transcription efficiency (Ho and Strauss, 2004; Shifera and Hardin, 2010). This information is presented as Supplementary Data.

We also evaluated the effect of the other extracts on hPXR transcription activity. We observed that *E. mulungu* is a hPXR partial agonist since it increased hPXR reporter gene



transcription activation in a dose dependent manner, but not as strong as rifampicin; *Lippia sidoides* did not show any effect on hPXR; *Cordia verbenaceae* failed to increase hPXR transcription activity. When we treated the cells with *Cordia verbenaceae* extract the transcription activity of rifampicin was impaired by 50% suggesting an antagonist effect. However, this is due to

unspecific effects, since inhibition was also observed with a high affinity thyroid hormone receptor (Supplementary Figure S16, Supplementary Data).

Modulation of Intracellular Glutathione Levels by Plant Extracts

As shown in Figure 3, *Solanum paniculatum*, *Lippia sidoides* and *Cordia verbenacea* led to significant reduction of intracellular glutathione levels. The declines seen in the cells treated with *Solanum paniculatum* and *Cordia verbenacea* are statistically comparable to BSO (10 μ M) ($p < 0.001$). *Lippia sidoides* also significantly inhibited the accumulation of glutathione in cells ($p < 0.05$). *E. mulungu*, on the other hand, was able to significantly increase the intracellular glutathione level ($p < 0.001$).

GGT Activity Is Compromised by *Cordia verbenacea*

The GGT activity in HepG2 cells was significantly lowered by *Cordia verbenacea* ($p < 0.01$). The GGT inhibitor acivicin was able to reduce its activity in a concentration of 5 μ M ($p < 0.001$) (Figure 4).

P-gp Efflux Activity Is Not Affected by the Plant Extracts

Overexpression of P-gp protein and the efflux activity in Caco-2 VCR cells are confirmed by the results previously reported by Eneroth et al. (2001) and by experiment conducted in our lab (Data presented as Supplementary Materials). None of the plant extracts could significantly modulate P-gp activity in Caco-2 VCR

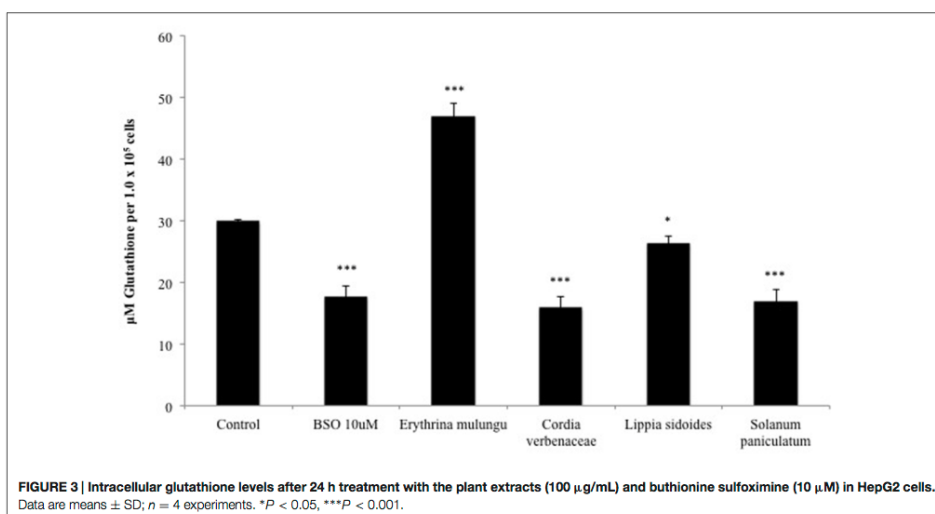
cells at the tested concentration. The P-gp inhibitor verapamil (20 μ M) was able to significantly impair the efflux of rhodamine 123 ($p < 0.001$) (Figure 5).

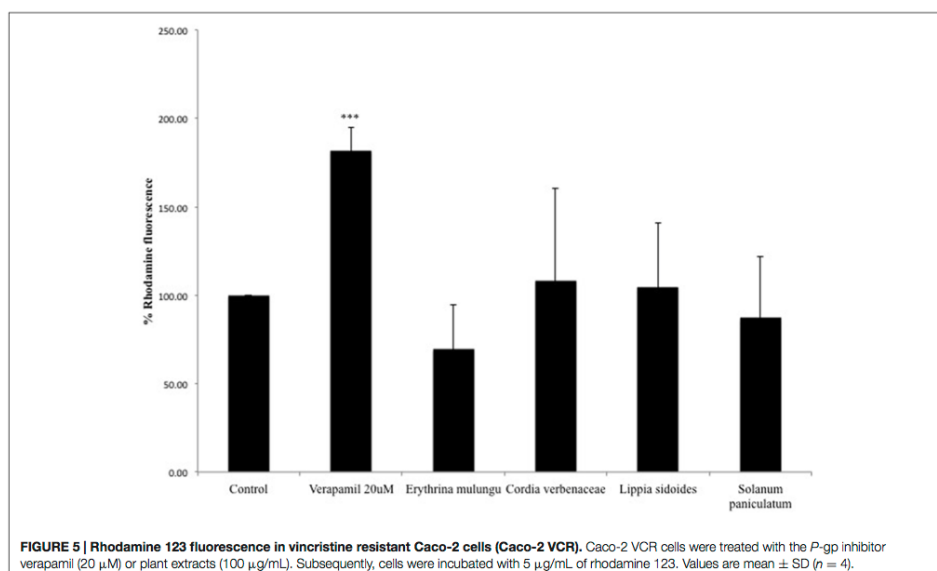
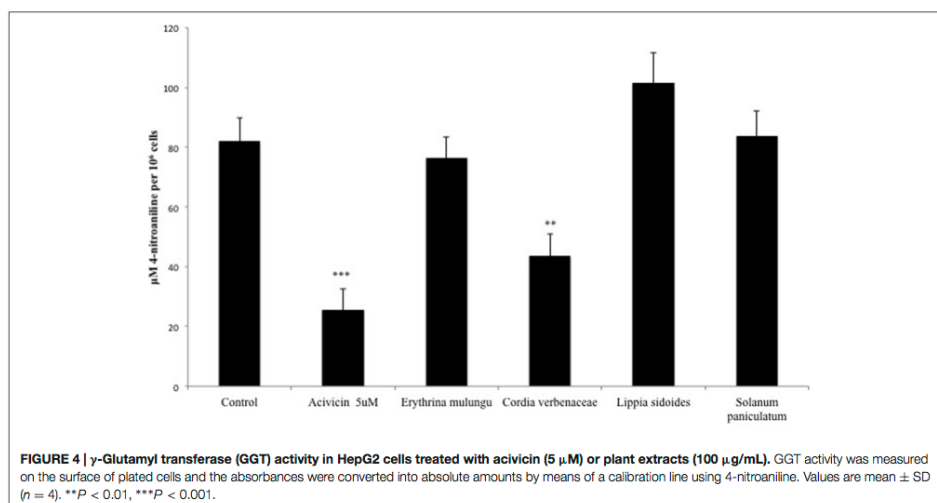
DISCUSSION

In Brazil, information about potential interactions caused by herbal medicines and conventional medicines alike, must be clearly described in the patient information leaflet. Besides HDI, other types of interactions should also be disclosed to the patient, such as drug-food, drug-chemical substance, drug-laboratory and non-laboratory test, and drug-disease interactions. Such data is available for newly registered herbal medicines, which have undergone preclinical and clinical tests. On the other hand, similar studies are not yet available for herbal medicines that are included in the ANVISA's traditional herbal medicine list. In this case, the regulatory approval from ANVISA is given without the need of those studies.

To help in this endeavor, we investigate whether the four selected Brazilian native medicinal plants can alter the activity and/or expression of discrete metabolic and/or transporter proteins in order to evaluate which prescription drugs (single chemical entities) would be affected by their concomitant use.

Solanum paniculatum was the only medicinal herbal drug that decreased CYP3A4 gene expression. We show that this down-regulation is due—at least in part—to an antagonistic effect of the plant extract on hPXR. CYP3A is the most relevant family of phase 1 metabolism and CYP3A4 is responsible for metabolizing more than 50% of marketed drugs (Wienkers and Heath, 2005). hPXR is a nuclear receptor that plays a key role in the regulation of xenobiotic-inducible CYP3A mRNA gene expression and most





cases of CYP3A4 gene regulation are related to the modulation of hPXR (Dong et al., 2010).

Our results must be put into the context of the limitations of HepG2 as a model for induction studies (Gerets et al., 2012).

Although less sensitive than human hepatocytes (HH), HepG2 line is able to express CYP3A4 mRNA under the conditions established by Sumida et al. (2000). The poor response to induction is overcome by the sensitivity of RT-PCR, and this

technique has been used for the study of natural products by several authors such as Cui et al. (2014) and Kumagai et al. (2016). Studies with HH should follow our screening to confirm its potential clinical significance (Sumida et al., 2000; Gerets et al., 2012; Cui et al., 2014; Kumagai et al., 2016). This could be the reason that we can see some antagonistic effects for *E. mulungu* in transfected HeLa cells but no modulation of CYP3A4 gene expression in HepG2 cells (See Supplementary Data).

Solanum paniculatum, together with *Cordia verbenacea* and *Lippia sidoides*, was also able to decrease intracellular glutathione levels, an important metabolite involved in the clearance of xenobiotics and the detoxification of reactive species (Xie, 2009). The variations of intracellular glutathione may be related to different effects, i.e., (a) inhibition/induction of enzymes involved in its biosynthesis, such as glutamate-cysteine ligase (GCL) and glutathione synthase (Lu, 2013); (b) the increase (or the inhibition) of GSH efflux from cells (Aw et al., 1986; Fernandez-Checa et al., 1988; Lu et al., 1990) which may be associated also with cell death (De Nicola and Ghibelli, 2014); (c) the formation (and the possible release) of GSH-adducts could also cause a reduction of the detectable intracellular glutathione (if it is not compensated by a new GSH synthesis) (Blair, 2010); finally (d) a modulation of glutathione transferase (GST) activity could also explain changes in both intra- and extracellular glutathione levels (Tolson and Wang, 2010). As for (a), the presence of active compounds in *Solanum paniculatum*, *Cordia verbenacea* and *Lippia sidoides* could be depleting glutathione in a similar manner BSO does, i.e., by inhibiting the GCL enzyme (Marengo et al., 2008). In order to identify these active compounds, a bioguided isolation strategy would be needed. Literature data reports that polyphenols such as gallic acid and derivatives, flavonoids such as luteolin, chrysin and apigenin, among others, induce GCL expression rather than inhibiting its activity (Panich et al., 2012; Huang et al., 2013). (b) is less likely to happen due to cell death as we are working with concentrations of plant extracts that are more than ten times lower than their maximum non-toxic concentration ($>1000 \mu\text{g/mL}$). More refined experiments would be needed to attest the formation of GSH-adducts, as stated in (c). As per (d) is another target that should be studied if the mechanism of glutathione depletion needs to be unveiled.

Depletion could be also linked to a non-functional γ -glutamyl cycle. This cycle serves as a continuous source of cysteine, which plays a key role in glutathione synthesis. GGT is an enzyme that first catalyzes the cleavage of glutathione on its γ -glutamyl-cysteine bond. Cysteinylglycine is then broken down by a dipeptidase to form glycine and cysteine. Cells reuptake those amino acids in order to synthesize more glutathione. Deficiency of GGT activity could potentially lower glutathione levels due to loss of cysteine (as glutathione) in the urine. Therefore, glutathione synthesis would be impaired due to the absence of this amino acid (Chevez-Barrios et al., 2000; Lu, 2013).

Indeed, the reduction of glutathione levels found in HepG2 cells treated with *Cordia verbenacea* turned out to be due—at least in part—by its ability to decrease GGT activity. However, *Solanum paniculatum* and *Lippia sidoides* did not affect GGT activity in a

significant way, so glutathione levels in those cases could have been affected via other mechanisms.

Dereplication of the active principles responsible for this activity can be attempted from our HPTLC analysis. The flavonoid luteolin was found in *Lippia sidoides* and gallic acid was found in *Solanum paniculatum*. Those compounds have been reported to deplete intracellular glutathione levels. A study published by Balyan et al. (2015) demonstrated that intracellular glutathione levels were significantly depleted by luteolin in the human melanoma SK-MEL-28 cell line. This was explained by an inhibition of glutathione S-transferase (GST) activity via competitive reversible and irreversible mixed mechanisms (Balyan et al., 2015). Another study published by Locatelli et al. (2009) showed that gallic acid was able to deplete intracellular glutathione levels in melanoma cells through inhibition of the activity of γ -Glutamyl-cysteine synthetase. Gallic acid has been also proven to inhibit GGT activity in mice (Mahajan and Mahmood, 2009).

Last but not least, *P-gp* activity was not affected by any of the extracts. This is a positive finding taking into consideration that this is one of the most important targets in preclinical studies.

As for *E. mulungu*, it did not affect any of the targets selected in this work, however, it increased glutathione levels. Although this effect is not harmful in itself, its ability to cause HDIs cannot be ruled out by helping to metabolize other drugs through the formation of GSH adducts. We should still warn that other types of interactions may be important in this case. In Brazil, this medicinal plant has been used for a long time as a natural sedative. Indeed there is *in vivo* data validating its diazepam-like activity, so caution is warranted when patients have to combine this with other central nervous system (CNS) active prescription medicines, drive or operate potentially dangerous machines at work (Onusic et al., 2002).

Brazilian regulations on herbal medicines have been changing over the years. Improving the regulatory framework for herbal medicines is necessary due to the increased number of unlicensed products on the market. ANVISA has a guide for such studies, called “Guide for the conduction of non-clinical toxicology studies and safety pharmacology required for the development of medicines” (Carvalho et al., 2014).

Information about HDI for the plants to be used by the Brazilian Health system is generally scarce. Those used worldwide, such as *Allium sativum* and *Cynara scolymus* have some interaction data already documented (Mazzari and Prieto, 2014a). Such information is even more scarce for the Brazilian native species, with *Paullinia cupana* as an exception (Rodrigues et al., 2012).

Evidence about HDI is, in most cases, based on alleged relations of the plant constituents with known mechanisms of action. For example, there are no published studies about potential HDI of *Maytenus ilicifolia*. However, it is known that quercetin and kaempferol are *P-gp* modulators found in the plant extract. Therefore, it is assumed but not attested, that HDI are likely to happen in this case (Zhou et al., 2004). As an example, we detected quercetin in our *Lippia sidoides* sample but such amount was not enough to cause changes in *P-gp* activity *in vitro*.

Patients in Brazil do not make exclusive use of registered herbal medicines manufactured by the industry. Local pharmacies are legally allowed to produce pharmaceutical products, which could contain a medicinal plant in the formulation. In this case, the final product does not require the approval of ANVISA to be sold to the patient. Furthermore, medicinal plants are also used in homemade preparations and in those cases data on interactions are even scarcer. ANVISA says that it is time to focus the research on Brazilian native plants in order to reveal the real interactions they could cause. This could improve the knowledge of users, health professionals and even public decision makers.

Experimental studies, such as preclinical studies, which are the subject of this report, are therefore highly important to elucidate underlying mechanisms. Those preclinical studies will serve as guidance for future clinical studies. Eventually, more potential cases of HDI could be prevented rather than being late diagnosed by the pharmacovigilance systems (De Smet, 2007).

CONCLUSION

This preclinical study evidences the possibility that the administration of some of these herbal medicines may be able to cause *in vitro* disturbances to metabolic mechanisms. More refined studies would be necessary to ascertain the *in vivo* and/or clinical significance of such interactions. At this point, we can suggest that active pharmacovigilance is recommended for *Cordia verbenaceae*, *Lippia sidoides*, and especially in the case of *Solanum paniculatum* for which an *in vitro* hPXR-mediated reduction of CYP3A4 gene expression accompanied by significant depletion of glutathione is suggested here for the first time.

Although further studies are needed in order to attest the clinical relevance of our findings, we hope that this work will contribute to stimulate similar research toward the regulation of the quality and safety of these herbal medicines in Brazil.

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AUTHOR CONTRIBUTIONS

AM contributed to the experimental design and was in charge and performing and setting up all the assays involving Caco-2 and HepG2 cell lines and the writing of the manuscript. SF contributed to HPTLC analysis and to the writing of the manuscript. FdN and FM contributed to the PXR studies. DS contributed by sourcing and extracting the plant material. AC contributed to the regulatory aspects of herbal medicines in Brazil. JP contributed to the experimental design and the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

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